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Hedgehog and Bmp polarise haematopoietic stem cell emergence in the zebrafish dorsal aorta

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Summary

Haematopoietic stem cells (HSCs) are first detected in the floor of the embryonic dorsal aorta (DA) and here we investigate the signals that induce the HSC programme there. We show that while continued Hedgehog (Hh) signalling from the overlying midline structures maintains the arterial programme characteristic of the DA roof, a ventral Bmp4 signal induces the blood stem cell programme in the DA floor. This patterning of the DA by Hh and Bmp is the mirror image of that in the neural tube, with Hh favouring dorsal rather than ventral cell types, and Bmp favouring ventral rather than dorsal. With the majority of current data supporting a model whereby HSCs derive from arterial endothelium, our data identifies the signal driving this conversion. These findings are important for the production of HSCs from embryonic stem cells and establish a paradigm for the development of adult stem cells.

Introduction

Blood and endothelium develop in close association, possibly from bipotential precursors called haemangioblasts (reviewed in Dzierzak and Speck, 2008). Bmp is critical for haemangioblast formation, and Hedgehog (Hh), VEGF and Notch are required for the specification of arterial endothelium and adult HSCs in zebrafish (Gering and Patient, 2005; Lawson et al., 2002; McReynolds et al., 2007; Walmsley et al., 2002). Subsequent to its earlier role in arterial and HSC specification, Hh is later required for continued expression of the transcription factor, *tbx20*, in the DA roof, proximal to the source of Hh in the notochord

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(Gering and Patient, 2005; Murayama et al., 2006; Zhang and Rodaway, 2007). This dorsal restriction of *tbx20* within the DA occurs at the same time as the initiation of definitive haematopoiesis in the ventral wall of the DA (Ahn et al., 2000; Gering and Patient, 2005) (Fig 1a), an observation which may reflect an antagonistic relationship between *tbx20* and the haematopoietic programme, as seen during the earlier development of embryonic blood (Szeto et al., 2002). Thus the restriction of HSC formation to the DA floor may be a consequence of continued Hh signalling.

While the location of Hh protein has not been determined in zebrafish due to a lack of available antibodies, *Hh* expression has been demonstrated within the dorsally located notochord, but not the ventrally located endoderm, during the time of HSC emergence (Krauss et al., 1993; Lewis et al., 1999; Roy et al., 2001). Perhaps the best readout for cells actively receiving Hh signalling is the expression of the receptor, Patched (Ptc), and *ptc1* is expressed within the dorsal wall of the DA, whilst *ptc2* exhibits more diffuse expression throughout the trunk, encompassing the entire DA (Lewis et al 1999). The distinct functions of these receptors are not well understood, but it is highly likely that they will have different targets, based upon the disparate phenotypes observed in *ptc1* and *ptc2* mutants (Koudijs et al., 2008; Koudijs et al., 2005). Lewis et al's data indicate that *ptc1* expression requires a higher level of Hh signalling than *ptc2*, consistent with its expression in the dorsal wall of the DA, proximal to the source of midline Shh. Thus it is likely that Hh signalling in the dorsal wall of the DA is mediated by Ptc1. The expression of *ptc2* within the ventral wall of the DA, more distal to the midline Shh source, implies that the ventral DA experiences a low level of Hh signalling. However, inhibition of Hh signalling at this time indicated that it is not required for HSC programming (Gering and Patient, 2005).

Here we demonstrate that distance from Hh signalling contributes to but is not solely responsible for the ventral localisation of HSCs. Using a conditional transgenic strategy, and in parallel, morpholino knock down, we provide *in vivo* functional evidence that *bmp4* is required for the *runx1*-mediated emergence and maintenance of HSCs in the ventral wall of the DA.

Results

Ectopic Hedgehog signalling expands *tbx20* expression into the ventral wall of the DA but does not suppress initiation of HSC gene expression

Because *tbx20* and HSC gene expression are mutually exclusive, we tested the possibility that Hh might restrict HSC formation, by exposing the DA floor to continued Hh signalling. To expand the range of Hh signalling, embryos were treated with purmorphamine (PMA), which activates the Smoothed receptor (Sinha and Chen, 2006). Treated embryos exhibited morphological and gene expression alterations characteristic of Hh pathway activation (Fig S1) (Concordet et al., 1996; Ekker et al., 1995). In embryos treated from the 15-somite stage, when maintenance of *tbx20* expression in the roof of the DA is Hh dependent, *tbx20* expression was expanded into the DA floor (Fig 1c, e, red arrows). At the times studied, the endothelium of the DA consists of a single cell layer thus, since *tbx20* staining directly abuts the DA lumen but also extends greater than one cell diameter ventrally (Fig 1e, red arrow), under the influence of PMA both endothelial cells of the DA and the ventral mesenchyme beneath were induced to express *tbx20*. Thus, elevated Hh signalling can induce *tbx20* expression in the floor of the DA, making its absence there normally a likely consequence of distance from the notochord source of Hh (Fig 1a).

To examine HSC programming, we monitored *runx1* expression, which is essential for HSC development and is the first sign of HSC formation in zebrafish (Burns et al., 2005; Gering and Patient, 2005; Kalev-Zylinska et al., 2002). Despite the ectopic expression of *tbx20* in

the DA floor, *runx1* expression in PMA treated embryos was indistinguishable from control embryos at 24hpf (Fig 1f-g, red arrows). Both the numbers of *runx1*⁺ cells and their levels of expression were unaffected. There was a similar lack of effect at 23hpf, when *runx1* expression in the DA is first detectable, and also at 25hpf and 26hpf (data not shown). We therefore conclude that Hh affects neither the initiation of *runx1* expression in the DA nor its maintenance.

It was not possible to study gene expression downstream of *runx1*, such as *cmyb* and *ikaros* (Gering and Patient, 2005; Thompson et al., 1998; Willett et al., 2001), since PMA treated embryos do not develop circulation due to loss of the axial vein (data not shown), and therefore the primitive blood expressing *cmyb* and *ikaros* remains in the ICM region, masking expression in the HSCs in the DA floor. Furthermore, it was not possible to assess derivatives of the HSCs in the thymus, for example *rag1* expression (see Fig 2j-k & Fig 4k-l), because absence of circulation prevents HSC migration to this region (Kissa et al., 2008; Murayama et al., 2006). However, the initiation and maintenance of an additional HSC marker, *gf1*, was also unaffected in the DA of PMA treated embryos, as seen for *runx1* (Fig S2). Taken together, the undisturbed initiation of *runx1* and *gf1* expression in PMA treated embryos indicates that distance from the Hh source cannot alone be responsible for the localisation of HSC emergence in the DA floor.

Bmp signalling is required for initiation and maintenance of *runx1* expression in the ventral wall of the DA

Analysis of gene expression in the zebrafish trunk revealed that Bmp antagonists surround the pocket in which blood and endothelium differentiate, but that just prior to HSC emergence, the environment is transformed into one favouring Bmp signalling ventral to the DA (Fig 2a). Thus, the pocket in which the blood and endothelium differentiate is surrounded by tissues expressing *chordin*, *noggin2* and *type II collagen* up to 22hpf (Furthauer et al., 1999; Yan et al., 1995) (and Fig S3). However, by 24hpf, while *noggin1* remains expressed in the ventral somite distal to the DA, the pronephric ducts (PNDs) in the anterior, and the ventral mesenchyme in the posterior, express *bmp4* (Chin et al., 1997) (and Fig S3a-b). In addition, the major vessels, including the DA, continue to express *tolloid* (from 21hpf), which cleaves residual Chordin (Connors et al., 1999). Furthermore, *bmpr2a*, *alk1*, *alk3*, *alk8* and *smad5* are all expressed in the DA (Monteiro et al., 2008). Thus, immediately prior to HSC emergence, the environment switches from anti- to pro-Bmp signalling, suggesting a potentially direct inductive role for Bmp in HSC emergence within the DA. Similar, localised expression of *bmp4* has been observed in human, mouse, chick and *Xenopus* embryos (A. Ciau-Uitz and RP, unpublished; Marshall et al., 2000; Pimanda et al., 2007; Suonpaa et al., 2005).

To functionally test such a role for Bmp signalling, we used a transgenic zebrafish line expressing a temperature-inducible, truncated Bmp receptor (tBR), which acts as a dominant-negative mutant (Pyati et al., 2006; Pyati et al., 2005). Because tBR is fused to GFP, fluorescent embryos expressing tBR can be separated after heat shock from control embryos derived from the same transgenic heterozygous parent. Embryos were heat shocked at 21hpf so that Bmp signalling would be inhibited by 23hpf when *runx1* expression initiates in the DA. Immunohistochemistry for phosphorylated Bmp receptor Smads confirmed that Bmp signalling was inhibited by 23hpf and recovering by 26hpf (Fig S4).

Embryos fixed between 23hpf and 26hpf were assayed for *runx1* expression (Fig 2b-i). At 23hpf, 20/22 GFP⁻embryos displayed *runx1*⁺ cells in the DA, whereas 15/22 GFP⁺embryos possessed no DA staining. In both GFP⁺ and GFP⁻embryos, *runx1* staining was detected equivalently in other areas of the embryo such as the olfactory placode and the Rohon-Beard neurons, demonstrating that the loss of *runx1* expression was DA specific (Fig S5). The

runx1 reduction in the DA persisted to 25hpf in the GFP⁺embryos, but by 26hpf *runx1* expression was recovering (Fig 2h-i). Thus, the loss of *runx1* in the DA correlates with downregulation of Bmp signalling at 23-25hpf, and recovery of *runx1* at 26hpf correlates with the turnover of tBR and the recovery of phosphorylated Smad activity (Fig S4).

The first clearly identifiable derivatives of the definitive lineage in zebrafish embryos are the *rag1/2* and *ikaros* expressing thymocytes in the bilateral *thymi* at 4dpf (Kissa et al., 2008; Willett et al., 1997), and we could test for the presence of these cells since all embryos possessed normal circulation. Consistent with a loss of HSCs in the DA, *rag1* expression in the same batch of embryos at 4dpf was reduced in GFP⁺embryos compared with GFP⁻embryos (Fig 2j-k). Furthermore, although analysis of additional HSC markers such as *cmyb*, *ikaros* or *cd41* was not possible since these genes are expressed in the overlying primitive blood at the time of HSC emergence (Gering and Patient, 2005; Lin et al., 2005; Thompson et al., 1998), the expression of *gfi1*, which is restricted to the definitive lineage, behaved in an identical manner to *runx1* following Bmp inhibition (Fig S6). Taken together, these results demonstrate that Bmp signalling is required for the initiation of the HSC programme in the DA and for normal levels of HSC descendants.

To determine if Bmp signalling is required for maintenance as well as initiation of the definitive haematopoietic programme, we performed a heat shock at 22hpf, and assayed for *runx1* expression (Fig 3a-h). GFP⁺(24/24) and GFP⁻(19/19) embryos both possessed strong *runx1* staining in the DA at 23hpf. However, at 24hpf, a strong downregulation of *runx1* expression was observed, which persisted until 26hpf. These results demonstrate that Bmp signalling is required both for the initiation and maintenance of *runx1* expression in the floor of the DA.

To determine if elevated Bmp signalling could expand the domain of *runx1* expression dorsally, we analysed the expression of *runx1* and *gfi1* in *dino* mutants, which possess a genetic lesion at the *chordin* locus (Fig S7) (Schulte-Merker et al., 1997). Surprisingly, the initiation and maintenance of both markers in the DA were unaffected in *dino* embryos and neither gene exhibited ectopic expression in the DA (Fig S7a-j, red arrows); however, both were ectopically expressed in the posterior ICM as previously demonstrated (Leung et al., 2005). Arterial expression of *tbx20* was unaffected, while its expression within a ventral population of cells under the yolk cell extension was substantially increased in *dino* mutants in comparison to wild-type embryos (Fig S7k-l, yellow and orange arrows, respectively), as expected for Bmp-dependent expression (Fig S8a-h, red arrows and Pyati et al., 2006). Preliminary experiments with a heat shock inducible Bmp2b transgenic zebrafish line (Chocron et al., 2007), support this lack of HSC gene expression in the dorsal DA (data not shown). We therefore conclude that Bmp levels may already be optimal ventrally and that dorsal cells may be unable to respond.

The arterial programme is unaffected by loss of Bmp signalling

Since arterial specification and HSC emergence are intimately linked (Burns et al., 2005; Gering and Patient, 2005), the loss of *runx1* in the DA led us to examine the arterial programme in Bmp-inhibited embryos. Arterial markers such as *notch5* and *deltaC* are expressed throughout the DA roof and floor from 22hpf, shortly before *runx1* expression (Patterson et al., 2005). We therefore induced tBR expression via a 21hpf heat shock and assayed arterial gene expression alongside *runx1*. We found no difference between GFP⁺ and GFP⁻embryos at any stage, even at stages when *runx1* expression was lost (Fig 3i-r and data not shown). Thus, the loss of *runx1* expression was not due to a loss of endothelium in the DA floor, but a loss of expression within those cells. Analysis of *tbx20* expression also revealed no differences between the GFP⁺ and GFP⁻embryos in expression or polarisation of *tbx20* transcripts (Fig S8). Taken together, these observations demonstrate that Bmp

signalling within the trunk prior to the onset of HSC gene expression is required for initiation of definitive haematopoiesis in the ventral wall of the DA but not the maintenance of ventral arterial marker expression in those cells.

***bmp4* is required for HSC emergence within the ventral DA**

Since induction of tBR produced only transient inhibition of Bmp signalling (Fig S4), which led to recovery of HSC gene expression (Fig 2b-i) and consequently only a moderate downregulation of *rag1* at 4dpf (Fig 2j-k), we wished to determine if a more complete loss of Bmp signalling could create a more profound effect on the HSC programme. Given the presence of *bmp4* transcripts proximal to the DA floor during the initiation of definitive haematopoiesis in zebrafish (Fig 2a and Fig S3a-b), and also in other vertebrates, together with the absence of *bmp2b* and *bmp7* transcripts in zebrafish (data not shown), we knocked down *bmp4* expression by morpholino (mo) injection (Chocron et al., 2007). Embryos injected with *bmp4* morpholino displayed inappropriate expression of *gata1* at 10s in the most ventral posterior mesoderm (Fig S9a-b), as previously demonstrated in a *bmp4* mutant (Stickney et al., 2007). At 26hpf, *bmp4* morphants exhibited complete loss of *runx1* expression within the DA (Fig S10a-b), however, neural *runx1* expression remained unaffected, demonstrating that *bmp4* is required for the initiation of *runx1* expression in the ventral wall of the DA. Furthermore, at 28hpf, *runx1* expression remained absent from the DA in two thirds of *bmp4* morphants (Fig 4a-d, arrows), while *cmyb* expression was also substantially downregulated in the DA of *bmp4* morphants (Fig 4e-h, red arrows). Interestingly, *cmyb* expression within the erythromyeloid progenitors (EMPs) in the posterior blood island was unaffected in *bmp4* morphants (Fig 4g-h, green arrows), supporting previous studies which indicate that EMPs arise independently of HSCs (Bertrand et al., 2007). While HSC gene expression was reduced, the expression of arterial markers was unaffected in *bmp4* morphants (Fig S9c-j), showing that the *bmp4* signal acts specifically on the definitive haematopoietic programme and not the maintenance of arterial gene expression within the DA.

To confirm that the morpholino-induced loss of *runx1* expression in the DA truly reflected a loss of HSCs, we assayed *rag1*⁺, *ikaros*⁺ thymocytes at 4dpf. *bmp4* morphants contained substantially reduced *rag1* and *ikaros* expression within the thymus, while expression of *foxn1* in the thymic epithelium was unaffected, demonstrating that the *thymi* were present but contained substantially fewer thymocytes (Figs 4i-l and S10c-f, arrows). To confirm that HSCs and not just T-cells were affected, we analysed the expression of *cmyb* within the developing kidney at 5dpf and found that *bmp4* morphants exhibited a substantial downregulation or total absence of *cmyb* in comparison to controls (Fig 4m-n, outlined). Taken together with the downregulation of *rag1* observed in embryos expressing tBR (Fig 2k-l), these data indicate that *bmp4* induces HSCs in the DA floor. Thus, overall, the data presented here show that dorsoventral patterning of the DA is a mirror image of the patterning of the neural tube, whereby Bmp specifies ventral rather than dorsal fate and Hh specifies dorsal rather than ventral cell fates (Fig 4o).

Discussion

The first HSCs in developing embryos are clearly located in the floor of the DA, however whether they arise from the wall of the DA, the underlying mesenchyme or the embryonic blood has been a matter for some considerable debate (Dzierzak and Speck, 2008). However, evidence supporting an endothelial precursor for the HSC continues to accumulate, most recently using VE-Cadherin to label the cells (Chen et al., 2009; Zovein et al., 2008). Furthermore, both cell lineage tracing and genetic disruption of signalling pathways support independent development of embryonic blood and HSCs, and the shared signalling dependence of the arterial and HSC programmes also support the DA wall as the

source of the HSCs (Ciau-Uitz et al, 2000; Burns et al., 2005; Gering and Patient, 2005). We have now identified two signals that differentially affect these two programmes, with Hh maintaining the dorsal arterial programme and Bmp inducing the ventral HSC programme.

In chick embryos, at the time of HSC emergence, the DA contains two different mesodermal contributions, with the floor derived from splanchnopleural mesoderm and the roof from somites (Pouget et al., 2006). It is formally possible therefore that the observed polarisation of the DA, with HSCs only arising in the floor, reflects cell lineage rather than local signalling. However, thus far in zebrafish, evidence has only been produced for a contribution to the DA from lateral (splanchnopleural) mesoderm (Zhong et al., 2001). In addition, more recent evidence from the chick indicates that even the roof of the DA initially derives from splanchnopleural mesoderm (T. Jaffredo, pers. comm.; Pouget et al., 2006). Thus, even in the chick the splanchnopleural mesoderm derived DA is likely to be patterned before its replacement by somitic endothelial cells. However, the failure to induce ectopic HSC gene expression in *dino* embryos, suggests that Bmp signalling may be optimal around the ventral DA and that the dorsal wall of the DA may not be competent to receive Bmp signalling.

The precocious differentiation of the posterior PLM into *gata1*+ primitive blood induced by *tbx20* knockdown can be mimicked by morpholino knockdown of *bmp4* (Fig S9a-b), since *tbx20* is under the control of Bmp signalling in these cells (Pyati et al., 2006). Conversely, in *dino* mutant embryos, which exhibit increased Bmp signalling, *tbx20* expression is expanded in the posterior PLM and *gata1* expression is reduced (data not shown). Thus, *bmp4* controls the timing of primitive haematopoietic differentiation in the posterior PLM via *tbx20*. However, since Hh-dependent *tbx20* expression in the DA does not antagonise definitive haematopoiesis, its presence there is likely to reflect its role in vascular lumenisation and inter-somitic vessel sprouting (Szeto et al., 2002). Furthermore, in contrast to its role in controlling the timing of primitive haematopoietic differentiation in the posterior PLM, *bmp4* expression around the DA is required to promote definitive haematopoietic specification by initiating the expression of *runx1* in the ventral wall of the DA.

Requirements for Notch and Hh signalling have been demonstrated for the expression of *runx1* within the DA, albeit at different times (Burns et al., 2005; Gering and Patient, 2005). Whilst a direct role for Hh signalling remains to be demonstrated, Notch signalling has been shown to be required directly for HSC gene expression in the DA at 36hpf (Burns et al., 2005). However, since we and others have shown that HSC gene expression is observed within the DA much earlier than this (Gering and Patient, 2005; Kalev-Zylinska et al., 2002), it remains to be determined whether Notch signalling acts directly in the initiation of definitive haematopoiesis within the DA. Therefore, it is formally possible that, whilst *bmp4* is responsible for initiation and early maintenance of the HSC programme, Notch signalling may be important in the later maintenance of the programme; however, parallel inputs of both pathways cannot be excluded.

The model proposed here for zebrafish seems likely to apply to other vertebrates since *bmp4* is expressed ventral to the DA in human, mouse, chick and *Xenopus* embryos (A. Ciau-Uitz and RP, unpublished; Marshall et al., 2000; Pimanda et al., 2007; Suonpaa et al., 2005). Furthermore, Bmp receptor Smads have been shown to transactivate the *runx1* promoter in a mouse myeloid cell line (Pimanda et al., 2007). Moreover, addition of Bmp4 to mouse AGM explants increases transplantable haematopoietic activity, although since in these experiments HSCs had already been formed, it was difficult to exclude an effect on expansion, as demonstrated for purified human haematopoietic progenitors (Bhardwaj et al., 2001; Durand et al., 2007). Thus, to the best of our knowledge our studies in developing zebrafish embryos represent the first demonstration that Bmp is required for HSC formation

in vivo. Together with the observation that Hh is required for maintenance of the arterial programme in the dorsal wall of the DA, the Bmp-dependent emergence of HSCs ventrally in the DA indicates that patterning of the DA has parallels with that of the neural tube (Fig 4o).

Experimental Procedures

Zebrafish husbandry

Zebrafish (*Danio rerio*) embryos were obtained from a wild type strain and raised at 28.5°C (Westerfield, 1993). Hemizygous transgenic embryos expressing under heat shock control a dominant negative Bmp receptor fused to GFP Tg(*hsp70l:dnBmpr-GFP*) or Bmp2b Tg(*hsp70l:bmp2b*) were used (Chocron et al., 2007; Pyati et al., 2005).

Heat shock inductions

Batches of transgenic embryos were strictly staged as described (Kimmel et al., 1995) to ensure minimal developmental asynchrony and subjected to a 43°C heatshock for 30 minutes at 21hpf or 22hpf as described (Pyati et al., 2005).

Purmorphamine treatments

Purmorphamine (Chemistry Research Laboratory, South Parks Rd, Oxford, United Kingdom; stock solution in DMSO: 2.5mg/ml) was used in aquarium water at 20µM. Embryos were treated from before MBT and from the 15 somite stage until collection.

In situ hybridisation

Performed as described (Gering and Patient, 2005). RNA probes were labelled with digoxigenin (Roche) and detected using BM Purple (Roche).

Wax sectioning

Embryos were sectioned in paraffin wax (Walmsley et al., 2002).

Antibody staining of phosphorylated Smad1, 5, 8

Embryos were fixed in 4% PFA in phosphate buffered saline (PBS) at 4°C, washed in ethanol and endogenous peroxidase activity was blocked by incubation in 0.5% normal goat serum (NGS) (Sigma)/0.5% H₂O₂ for 30min at 25°C before blocking in 10% NGS in PBS Tween® (Sigma) for 2 hours at 25°C. Incubation with pSmad1/5/8 antibody (Cell Signalling Technology) (1:200) was overnight at 4°C. Incubation with secondary antibody (goat α rabbit – HRP 1:200) (Vectorlabs) was for 2 hours at 25°C. Staining was developed using 3, 3'-diaminobenzidine (DAB) (Vectorlabs SK4100) as per manufacturers instructions. Embryos were re-fixed at 4°C in 4% PFA.

bmp4 MO injections

0.5nl *bmp4* morpholino (2ng/nl dissolved in distilled water) (Gene Tools) 5'-gggttttgattgctgacctcatg-3' (Chocron et al., 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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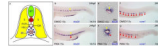


Figure 1. Ectopic Hedgehog signalling expands *tbx20* expression into the ventral DA but does not suppress the initiation of HSC gene expression

a) Diagram of transverse section illustrating DA polarisation in the zebrafish trunk at 24hpf.
b-g) Embryos treated with DMSO or PMA from 15S and probed for *tbx20* or *runx1* at 24hpf. Dorsal and ventral DA expression (yellow and red arrows). PMA treatment expands *tbx20* expression into the ventral DA (**c, e** compared to **b, d**) but *runx1* is unaffected (**f, g**).
AV, axial vein; **DA**, dorsal aorta; **NC**, notochord; **NT**, neural tube; **PMA**, purmorphamine; **S**, somite.

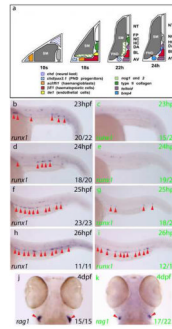


Figure 2. Bmp signalling is required for initiation of the HSC programme in the ventral DA
a) Gene expression summary as progenitors migrate to the midline and form blood and DA/AV endothelium. DA region becomes conducive to Bmp signalling by 24hpf. **b-i)** Expression of *runx1* (arrows) in embryos heat shocked at 21hpf. *runx1* expression in the DA at 23-26hpf (GFP⁻, black letters) was lost in GFP⁺embryos (green letters) at 23 and 24hpf. Representative embryos are depicted and numbers with or without *runx1* staining were scored. **j, k)** *rag1* expression in thymi at 4dpf heat shocked at 21hpf. 17/22 GFP⁺ embryos had reduced *rag1* expression compared to GFP⁻embryos, in which *rag1* staining showed little variation. The remaining 5/22 GFP⁺ embryos exhibited wild type *rag1* expression. **AV**, axial vein; **BL**, primitive blood; **DA**, dorsal aorta; **HC**, hypochord; **NC**, notochord; **NT**, neural tube; **PND**, pronephric duct; **SM**, somitic mesoderm.

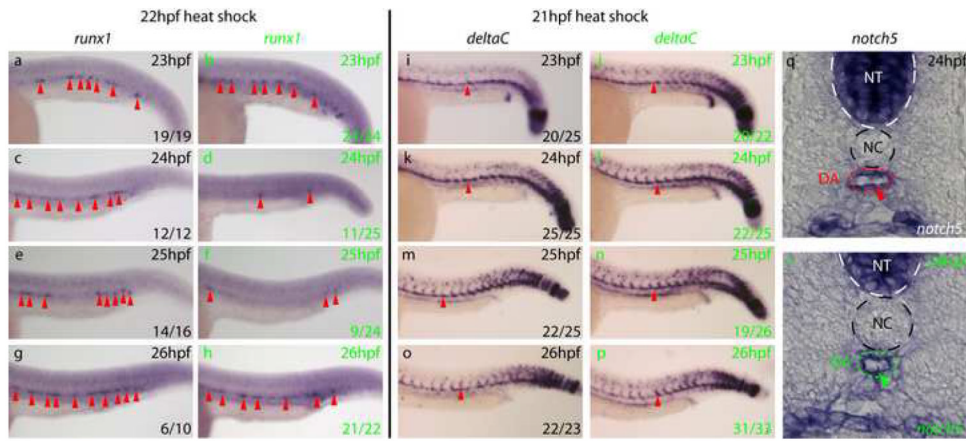


Figure 3. Bmp signalling is required for maintenance of *runx1* expression in the ventral DA but not for maintenance of the arterial programme
a-h *runx1* expression (arrows) in embryos heat shocked at 22hpf. *runx1* initiated normally, was lost by 24hpf in GFP⁺embryos (green letters) compared to GFP⁻embryos (black letters) but recovered by 26hpf. Representative embryos are shown. Embryos containing a few *runx1*⁺ cells were scored as negative.. **i-p** *deltaC* expression in the DA (arrows) in embryos heat shocked at 21hpf was unaffected by inhibition of Bmp signalling. **q, r** *notch5* expression in the ventral wall of the DA (arrow) in sectioned embryos heat shocked at 21hpf was normal.

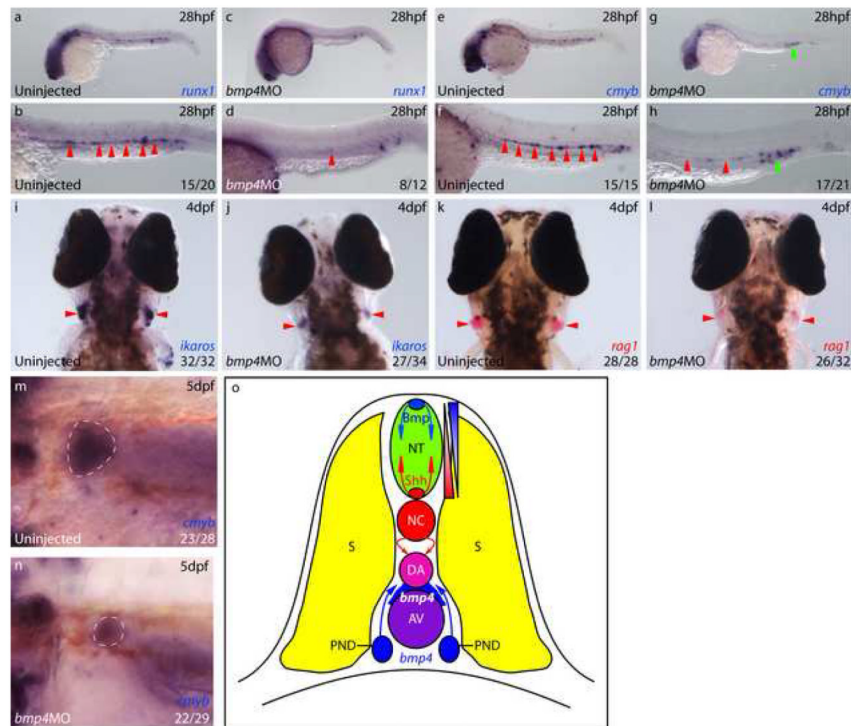


Figure 4. *bmp4* is required for HSC emergence within the ventral DA

a-d) *runx1* expression was absent from the DA (arrows) in 8/12 *bmp4* morphants at 28hpf (c, d), whilst the remaining 4/12 (a, b) exhibited reduced expression compared with uninjected controls. **e-h)** 17/21 *bmp4* morphants exhibited absent or substantially downregulated *cmyb* expression in the DA (red arrows) at 28hpf. **i, j)** At 4dpf, 27/34 *bmp4* morphants exhibited substantial downregulation of *ikaros* expression within the developing *thymi*, (arrows). The remaining 7/34 exhibited wild type expression. **k, l)** At 4dpf, 26/32 *bmp4* morphants exhibited substantially reduced expression of *rag1* in developing *thymi* (arrows), of which 7 exhibited no *rag1* expression. The remaining 6/32 were wild type. **m, n)** Dorsolateral views of the developing pronephros at 5dpf depicting substantial downregulation of *cmyb* expression (outlined) in 22/29 embryos, of which 4/29 exhibited no expression. The remaining 7/29 were wild type. **o)** Diagram of zebrafish trunk depicting polarisation of the DA by dorsal Hedgehog and ventral Bmp signalling, mirroring the neural tube.