Adult and embryonic blood and endothelium derive from distinct precursor populations which are differentially programmed by BMP in *Xenopus*

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SUMMARY

Blood and blood vessels develop in close association in vertebrate embryos and loss-of-function mutations suggest common genetic regulation. By the criteria of co-expression of blood and endothelial genes, and lineage tracing of progeny, we locate two distinct populations of progenitors for blood and endothelial cells in developing *Xenopus* embryos. The first population is located immediately posterior to the cement gland during neurula stages and gives rise to embryonic blood and vitelline veins in the anterior ventral blood island (aVBI), and to the endocardium of the heart. The second population resides in the dorsal lateral plate mesoderm, and contains precursors of adult blood stem cells and the major vessels. Both populations differentiate into endothelial cells in situ but migrate to new locations to differentiate into blood, suggesting that their micro-environments are unsuitable for haematopoietic differentiation. Both require BMP for their formation, even the Spemann organiser-derived aVBI, but individual genes are affected differentially. Thus, in the embryonic population, expression of the blood genes, SCL and GATA2, depend on BMP signalling while expression of the endothelial gene, Xfli1, does not. By contrast, Xfli1 expression in the adult, DLP population does require BMP. These results indicate that both adult and the anterior component of embryonic blood in *Xenopus* embryos derive from populations of progenitors that also give rise to endothelial cells. However, the two populations give rise to distinct regions of the vasculature and are programmed differentially by BMP.

Key words: Blood, Endothelium, Haemangioblast, BMP, Transcription, *Xenopus*

INTRODUCTION

Blood and endothelial cells are the first differentiated cell types to form in the developing vertebrate embryo, in response to mesoderm induction, and they are found in very close association in the extra embryonic mesoderm of the yolk sac (reviewed by Baron, 2001). It has been suggested that they derive from a common progenitor, called a haemangioblast (Murray, 1932), a suggestion consistent with the observation that many genes are co-expressed and/or required during early stages of blood cell and blood vessel development, including Flk1, cloche, SCL, QH1, Ets, Runx1, Fli1, TGF-β1, VEGF, CD34, Fli1, Tie2, GATA2, LMO2 and PECAM1 (Baron, 2001; Keller, 2001). Yet more compelling evidence is presented by the transient existence of embryonic stem cell-derived blast colonies that give rise to both lineages (Choi et al., 1998). Comparable colonies have been detected in cultures from dissected mouse embryos, suggesting that haemangioblasts may also exist in vivo (Nishikawa et al., 1998; Palis et al., 1999). However, final proof of the existence of the haemangioblast awaits single cell labelling in vivo.

The origins of blood in vertebrate embryos have been controversial. Evidence has been produced supporting both yolk sac and intra-embryonic origins for the adult blood stem cell (Keller, 2001). Transplantation studies in *Xenopus* embryos have demonstrated that the precursors of adult and embryonic blood are located in the dorsal lateral plate (DLP) mesoderm and the ventral blood island (VBI), respectively, at neurula stages (Kau and Turpen, 1983). Early fate maps, which concentrate on primitive (embryonic) blood, traced it to a region of the gastrula embryo called the ventral marginal zone (VMZ), although a small dorsal contribution was reported but ignored (Dale and Slack, 1987; Moody, 1987). Confirmation that embryonic blood also derives from dorsal marginal zone (DMZ) cells came from the study of the transcription factor Xaml, the *Xenopus* homologue of the haematopoietic progenitor gene, AML1/Runx1, which is normally expressed in the VBI at tadpole stages (Tracey et al., 1998). This study showed that isolated DMZ explants are able to express Xaml and that, in the intact embryo, when Xaml expression was suppressed in the DMZ by injection of a dominant negative form, the anterior blood island no longer expressed the blood marker α-globin. Subsequently, the fate map was redrawn by Lane and Smith, who looked at the contributions of 32-cell stage blastomeres to primitive circulating blood, monitored at later stages, and found that all blastomeres around the meridian of the embryo (tiers C and D) contributed to primitive blood (Lane and Smith, 1999). These authors, however, used
irregularly cleaving embryos, and a subsequent refinement of this fate map using regularly cleaving embryos showed that primitive blood in the anterior VBI derives from the dorsal blastomeres, C1 and C4, while the posterior VBI primitive blood arises from the ventral D4 blastomere (Ciau-Uitz et al., 2000). This study was the first to monitor the origins of the adult DLP blood and, importantly, found that this derives from the dorsal D3 blastomere. Although a very recent study using a different lineage tracer has raised the possibility that the original lacZ tracer may have labelled only a subset of the derivatives of the C3 blastomere (Lane and Sheets, 2002), the ability to label the adult and embryonic blood compartments separately demonstrates that their origins are distinct in *Xenopus*, whether at the 32-cell stage or slightly later.

The blood in the adult and embryonic compartments develops in close association with the endothelium of the early vasculature in *Xenopus* embryos. Expression of blood (SCL and GATA2) and endothelial (Xfli1) markers is detected in the DLP during tailbud stages, although double labelling to confirm co-expression has not been carried out (Ciau-Uitz et al., 2000). At stage 27 (tailbud), a subpopulation of these cells not expressing blood genes begins to migrate to the midline towards the hypochord, an endoderm-derived structure that secretes VEGF, where they form the dorsal aorta (Cleaver and Krieg, 1998; Ciau-Uitz et al., 2000). One day later blood gene expression is once again detected in and beneath the floor of the dorsal aorta (Ciau-Uitz et al., 2000). Lineage labelling of the C3 blastomere shows that derivatives of this blastomere are found in the DLP at tailbud stage and then in the dorsal aorta, as well as in mesenchymal cells below the dorsal aorta, suggesting that the haematopoietic cells associated with the floor of the dorsal aorta, thought to be the first adult blood stem cells, derive from the DLP (Ciau-Uitz et al., 2000). No similar analysis of blood and endothelium in the embryonic (VBI) compartment has been carried out.

The distinct origins and migration pathways of embryonic and adult blood suggest that they may come under the influence of different embryonic signals during their ontogeny. Indeed it has been demonstrated that ventral mesoderm transplanted into the DLP region contributed to adult haematopoiesis, and, conversely, that DLP mesoderm transplanted to the VBI region contributed to embryonic blood (Turpen et al., 1998). These results indicate that haematopoietic precursors located in both the VBI and the DLP have the potential to produce both blood lineages, and suggest that micro-environmental signals restrict differentiation into one or other of the lineages. The close association of blood and endothelial precursors suggests that endothelium may also respond to similar signals.

BMP4 has long been associated with VBI induction and the formation of blood. Thus, ectopic expression of BMP4 in intact *Xenopus* embryos, or in explants of prospective ectoderm, stimulates blood cell and globin production (Dale, 1992; Jones et al., 1992; Maeno et al., 1994b), and injection of a dominant-negative receptor for BMP results in a reduction of blood and globin (Graff et al., 1994; Maeno et al., 1994a). The role of BMP in endothelial development has not been studied in the embryo. The distinct origins of the blood compartments in *Xenopus* has allowed us to investigate their requirements for BMP signalling by targeting the relevant regions of the early cleaving embryo. Using endothelial markers as well as those for blood, in conjunction with lineage tracing, we show that both blood compartments derive from populations of cells that co-express blood and endothelial genes, which also give rise to the vasculature, and we have looked at their dependence on BMP signalling. We conclude that while all blood and endothelial development requires BMP, the specific molecular responses to this signal differ between the adult and embryonic compartments.

**MATERIALS AND METHODS**

**Embryo manipulation and microinjections**

*Xenopus* embryos were obtained and cultured as described (Walmsley et al., 1994), and staged according to Nieuwoop and Faber (Nieuwoop and Faber, 1967). RNA for embryo injection was obtained as described (Ciau-Uitz et al., 2000). Dominant-negative BMP receptor RNA (tBR) or nls β-gal RNA was injected into VMZ and DMZ regions of the four-cell stage embryos in a volume of 4 nl per blastomere or into single blastomeres of the 32-cell stage embryo in a volume of 1 nl. For single blastomere injections, nomenclature of each blastomere used was that of Nakamura and Kishiyama (Nakamura and Kishiyama, 1971). For the details of constructs used to prepare RNA for embryo injections see nls β-galactosidase (Harland, 1991) and tBR (Northrop et al., 1995).

**β-gal staining and in situ hybridisation procedures**

For β-gal analysis, embryos were fixed and stained as previously described (Ciau-Uitz et al., 2000). Whole mount in situ hybridisation was performed according to Bertwistle et al. (Bertwistle et al., 1996). When double in situ hybridisation was performed, embryos were hybridised with probes differentially labelled with fluorescein and digoxigenin. The first colour was developed using BCIP (Boehringer Mannheim) then BM Purple (Boehringer Mannheim) was used for the second colour reaction. The gene with the strongest expression was always chosen for first staining. In situ hybridisation to wax sections was performed according to Ciau-Uitz et al. (Ciau-Uitz et al., 2000). For the details of the preparation of labelled probes see the following publications: GATA2 and αT4-globin (Walmsley et al., 1994); GATA3 (Bertwistle et al., 1996); Xaml (Tracey et al., 1998); XHhex (Jones et al., 1999); Xfli1 (Meyer et al., 1995); SCL (Mead et al., 1998); Xlim1 (Taira et al., 1994); Xmsr (Devic et al., 1998); Nkx2.5 (Tonissen et al., 1994); and CG-1 (Sive et al., 1989).

**Histological analysis**

For histological analysis embryos were embedded in wax and 10 μm sections cut in a microtome as described (Ciau-Uitz et al., 2000). Sections of whole mounted embryos (10 μm) were obtained by wax embedding and cutting in a microtome as indicated previously (Ciau-Uitz et al., 2000). Sections were dewaxed and rehydrated to water through an ethanol series, counterstained for 30 minutes with 0.1% Eosin Y in 70% ethanol, dehydrated to 100% ethanol, cleared in xylene and finally mounted in DPX or mounted in 80% glycerol without counterstaining. For cryostat sectioning, β-gal stained embryos stored in 100% methanol were rehydrated to PBS-Tween through a methanol series. After rehydration, embryos were washed twice with 30% sucrose in PBS for 30 minutes before an overnight infiltration in OCT:30% sucrose in PBS (1:4). Embryos were then placed in plastic embedding moulds containing fresh OCT:30% sucrose in PBS (1:1), oriented and snap frozen in liquid nitrogen. Frozen blocks were attached to cryostat holders and 10 μm sections cut in a cryostat. Sections were collected on 3-amino-pyridoxy-silane coated slides and left to dry overnight. Sections were washed three times in PBS-Tween for 5 minutes and then mounted in 80% glycerol.

**Ribonuclease protection assay analysis**

RNA was prepared from frozen embryos using the SV Total Isolation
**RESULTS**

**Locations of cells co-expressing blood and endothelial genes**

Staining of *Xenopus* tail bud embryos for expression of the haematopoietic gene, SCL (Mead et al., 1998), reveals two major populations of cells (Fig. 1A, stages 24 to 28). These are the well-described two blood compartments, the VBI (ventral blood island, Fig. 1, red arrows) and the DLP (dorsal lateral plate, Fig. 1, black arrows), which give rise to embryonic and adult blood, respectively. The same two populations of cells appear to be detected when probing for expression of the endothelial gene, Xfli1 (Meyer et al., 1995) (Fig. 1B, stages 24 to 28), with expression in the future DLP apparent from stage 20 (Fig. 1B, stage 20b, arrows). However, a closer look at the VBI region by staining for expression of both genes in the same embryo, reveals that their expression is largely mutually exclusive, with Xfli1-expressing cells (purple, green arrows) surrounding the SCL expressing cells (turquoise, red arrows), prefiguring the locations of the vitelline veins (Vit) and the blood, respectively (Fig. 1C, stages 26a and 26b). ‘Blood island’ formation, with blood cells surrounded by endothelial cells, is also observed in the mouse yolk sac (Haar and Ackermann, 1971). In contrast to the VBI, the DLP at stage 26 appears to contain cells co-expressing the two genes (Fig. 1C, black arrows, stages 26a and b). As the DLP region gives rise to both adult blood and the endothelium of the major vessels, these cells may represent precursors of these two lineages.

At earlier stages of development, co-expression of SCL and Xfli1 is also detected in a group of cells located immediately posterior to the cement gland (Fig. 1C, stages 17, 20 and 22 and see Fig. 1A,B, stages 17 and 20a for expression of each gene alone). The overlap of SCL and Xfli1 expression is greatest at stage 17, with cells expressing only SCL (turquoise, arrowheads) becoming increasingly separated from the Xfli1 expressing cells (purple) as development proceeds (Fig. 1C, stages 17 and 20a, anterior views; 20, 22b ventral views; 20b, dorsal view; 22a, 23, 24, 26, 28 and 31, lateral views. Anterior is towards the left with exception of 17 and 20a. (A) Time course from stage 17 to 28 showing SCL expression. Notice that SCL expression in the DLP is first detected at stage 24 (black arrow). (B) Time course from stage 17 to 28 showing Xfli1 expression. Notice that Xfli1 expression in the DLP is first detected at stage 20 (black arrows). (C) SCL (turquoise) + Xfli1 (purple) whole-mount double in situ hybridisation in early *Xenopus* embryos. Black arrowheads indicate SCL + Xfli1 cells. Green arrows indicate the developing vitelline veins (Vit) surrounding the VBI (red arrows). NF, neural fold; CG, cement gland.
stages 17-22b). The anterior VBI, which contains the anterior components of the embryonic blood and associated vitelline veins, derives from the mesoderm of the dorsal marginal zone of the early gastrula embryo (Ciau-Uitz et al., 2000; Lane and Smith, 1999; Tracey et al., 1998). These cells travel the length of the embryo during gastrulation and enter the anterior VBI after passing behind the future cement gland. Thus, the cells detected posterior to the cement gland at stage 17, co-expressing SCL and Xfl1, may well be progenitors for the blood and endothelial cells of the anterior VBI.

Cells co-expressing blood and endothelial genes in stage 17 embryos are likely progenitors for the anterior VBI

In order to further characterise this putative aVBI progenitor population, we probed for additional genes expressed in blood [GATA2 (Walmsley et al., 1994) and Xaml (Tracey et al., 1998)] or endothelium [Xhex (Jones et al., 1999)], either singly (Fig. 2A, parts a,d,e) or in combination (Fig. 2A, parts f and g) and found that all these markers were expressed in the region immediately posterior to the cement gland. To explore this population at higher resolution, we carried out in situ hybridisation on 10 μm sections of neurula stage embryos with the same battery of probes (Fig. 2B). All of these genes are clearly expressed in the mesodermal layer immediately posterior to the cement gland (CG, Fig. 2B, part a) and marked by expression of the gene CG1 (Sive et al., 1989) (Fig. 2B, part g). GATA2 is also expressed in the endodermal and ectodermal layers at this stage (Fig. 2A, part a, Fig. 2B, part f), while XHex is also expressed in the liver diverticulum (Fig. 2B, part e). However, the locations of the positive signals for all of these genes suggest a significant degree of overlap (Fig. 2Bi), which is borne out by double-labelled in situ hybridisation (Fig. 2B, parts f, g) and hybridisation to alternate sections (Fig. 2B, parts b-h). Thus, all the blood and endothelial genes tested are co-expressed in the mesodermal layer immediately posterior to the cement gland at stage 17 (orange cells in Fig. 2B, part i). Anterior to these cells are a small group of cells expressing all the blood and endothelial genes except XHex (green in Fig. 2B, part i), while posteriorly there are cells expressing only blood genes (GATA2, SCL, Xaml; red in Fig. 2B, part i).

In order to explore the fate of these cells in this region, we carried out lineage tracing experiments. The progeny of the β-gal RNA-injected D1 blastomere of the 32-cell embryo (Fig. 2C, part a) populate the anterior VBI of tail bud (stage 26) embryos (Ciau-Uitz et al., 2000), leading to overlap between β-gal staining and blood and endothelial gene expression in all embryos examined (Xfl1 and SCL, 12/12 embryos; XHex, Xaml and αT4-globin, nine out of nine embryos; data not shown). At earlier stages of development, β-gal-labelled derivatives of the D1 blastomere are found posterior to the cement gland in the position of the cells co-expressing blood and endothelial genes (Fig. 2C, parts b,c, arrow). The other D1 derivatives are predominantly endoderm, including the liver diverticulum. The more anterior medial cells express Nkx2.5 (data not shown) but none of the blood or endothelial genes, making them unlikely contributors to the VBI. Thus, the cells co-expressing blood and endothelial genes that are located posterior to the cement gland in stage 17 embryos very probably represent progenitors of the anterior VBI cells expressing blood or endothelial genes at stage 26. Similar results were obtained for blastomere C1, which also contributes to the aVBI (Ciau-Uitz et al., 2000): the only lineage labelled cells co-expressing blood and endothelial genes were the putative progenitor population posterior to the cement gland (data not shown). To confirm that anatomically identifiable blood and endothelial cells are indeed formed from D1-labelled cells, we monitored embryos at much later stages when vasculogenesis has taken place and blood circulation has commenced. β-gal staining was found quite clearly in both endothelial cells of the vasculature (Fig. 2C, parts d,e, arrowheads) and in circulating blood cells (Fig. 2C, part e, arrow) in seven out of seven embryos in each case. Labelling was also detected in endocardium in all these embryos (data not shown). Thus, the extensive co-expression of endothelial and haematopoietic genes in the lineage labelled cells at stage 17, together with the later appearance of lineage label in blood and endothelial cells and their precursors, suggest that the cells posterior to the cement gland are progenitors of the blood and vasculature of the aVBI and also heart-associated vessels.

Cells co-expressing blood, endothelial and pronephric duct genes in the DLP at tailbud stages are likely progenitors of adult blood and the major vessels

Previous lineage labelling studies have shown that the cells of the DLP region can be labelled separately from the VBI by injection of β-gal RNA into the C3 blastomere of the 32-cell stage embryo (Ciau-Uitz et al., 2000). The lineage label is later found in the endothelial cells of the dorsal aorta (see also Fig. 3A, arrowhead) and the posterior cardinal veins (Ciau-Uitz et al., 2000). That the DLP gives rise to the dorsal aorta is supported by direct labelling of the DLP region (Cleaver and Krieg, 1998). C3-injected lineage label is also found in sub-aortic mesenchymal cells expressing blood genes (Ciau-Uitz et al., 2000) and in clusters of cells attached to the floor of the dorsal aorta (Fig. 3A, arrow). Such cells are currently the best candidates for the first adult haematopoietic stem cells developing in the aorta (Keller, 2001). Thus, the DLP region contains progenitors of both blood and endothelium. Cells in the DLP region co-expressing haematopoietic and endothelial genes would be strong candidates for these progenitors. However, thus far, co-expression has only been explored for one blood gene and one endothelial gene. We therefore set out to determine the extent to which co-expression could be observed in this region with a view to defining more precisely the putative progenitors there. The close proximity of the pronephric duct cells and their derivation from the C3 blastomere (Ciau-Uitz et al., 2000) led us to monitor genes expressed in these cells also.

The genes monitored were SCL and GATA2 for blood, Xfl1, XHex and Xmsr for endothelium and Xlim1 and GATA3 for pronephric duct. A careful time course of expression in the DLP region showed that Xlim1 is present in a broad area, including the DLP from neurula stages with DLP-restricted Xfl1 and GATA3 expression detected from stage 20, followed by GATA2 from stage 22, and then SCL, XHex and Xmsr from stage 24 (Fig. 3B). The blood genes, SCL and GATA2 become undetectable in the DLP region after stages 33 and 34, respectively, while the endothelial and pronephric genes continue to be expressed beyond stage 36, reflecting the
Fig. 2. Characterisation of the embryonic/aVBI progenitor population. Blood and endothelial genes are co-expressed in the anterior ventral mesoderm of the mid-neurula stage embryo. Expression of blood and endothelial genes in the ventral mesoderm of the stage 17 embryo was analysed by whole-mount in situ hybridisation (A) and in situ hybridisation on sections (B). Lineage labelling experiments (C) indicate that the blastomeres of the 32-cell stage embryo, which give rise to the aVBI, also give rise to the anterior ventral progenitor population. (A) Single whole-mount in situ hybridisation analysis of the expression of GATA2 (a), Xfli1 (b), SCL (c), Xaml (d) and XHex (e); and double whole-mount in situ hybridisation analysis of the expression of SCL + Xfli1 (f) and SCL + XHex (g) in the stage 17 Xenopus embryo. Anterior views in all cases with dorsal towards the top. Black arrows indicate SCL + Xfli1 - cells in f and SCL + XHex - cells in g. (B) Expression of Xfli1 (b), SCL (c), Xaml (d, h), XHex (e), GATA2 (f) and the cement gland marker XCG1 (CG-1, g) in the ventral mesoderm of the stage 17 Xenopus embryo. (a) A sagittal section of the stage 17 embryo, red square indicates the area shown in photographs. (i) Summary of gene expression analysis. In situ hybridisation was performed on 10 μm sequential sections. (b-f) Sections from a single embryo and (g-h) sections from a second embryo. Anterior is towards the left and ventral is towards the bottom in all cases. (C) Lineage trace showing the origins of the ventral progenitor population and its fate. (a) Representation of the 32-cell stage embryo with the D1 blastomere highlighted in blue. (b) Embryo showing localisation of D1 progeny at stage 17 of development, anterior view with dorsal towards the top. (c) Sagittal section (10 μm) of stage 17, D1 β-gal injected embryo. Red arrow indicates β-gal positive cells located in the anterior ventral mesoderm (blood and endothelial progenitors). (d,e) Transverse 10 μm wax sections showing localisation of D1 progeny at stage 41. Lineage trace is seen in a mature vessel, the ventral aorta (red arrowhead, d and e), and in circulating blood cells within the vessel (red arrow, e). Lineage label was also seen in the endocardium and vitelline veins in seven out of seven embryos. ARCH, archenteron; CG, cement gland; NF, neural fold.
formation of the posterior cardinal veins and the pronephric ducts in this location.

Relative spatial expression of the various genes in tailbud embryos was established by double in situ hybridisation (Fig. 3C). Whole-mounted embryos were sectioned transversely at two points in the anteroposterior axis posterior to the forming pronephros. Wholemounts, anterior sections and posterior sections are presented left to right for each probe combination. Probe combinations that could be used were restricted by the fact that only strong hybridisation signals could be detected using the BCIP technique (turquoise, see Materials and Methods). Thus, panels a-l and bb-dd were hybridised first with Xlim1, the pronephric marker (turquoise) and secondly (purple), with endothelial (Xfli1, XHex, Xmsr), blood (SCL)
Fig. 3. Characterisation of the DLP progenitor population. (A) The dorsal aorta and intra aortic haematopoietic clusters derive from the C3 blastomere. Transverse 10 μm wax section through the trunk at the level of the pronephric duct of a stage 43 embryo that had been injected with 250 pg β-gal RNA in the C3 blastomere at the 32-cell stage. At stage 43, β-gal is located in the endothelial wall of the dorsal aorta (red arrowhead) and in clusters of blood stem cells on the floor of the aorta (red arrow). Dorsal is towards the top. Black arrowheads indicate the pronephric duct; n, notochord. (B) Timing of gene expression in the DLP. Expression profiles were obtained by analysis of embryos subjected to whole-mount in situ hybridisation. Arrows indicate expression before stage 18 (Xlim1) or after stage 36 (Xlim1, Xfli1, GATA3, Xmsr and XHex). (C) Analysis of gene expression in the DLP of the stage 26 embryo. Embryos were subjected to whole-mount double in situ hybridisation and then 10 μm transverse wax sections cut at the level of the pronephric duct in order to analyse gene expression. (a-c) Xlim1+Xfli1; (d-f) Xlim1+SCL; (g-i) Xlim1+XHex; (j-l) Xlim1+GATA3; (m-o) Xfli1+SCL; (p-r) GATA3+Xfli1; (s-t) GATA3+SCL; (u-v) GATA3+XHex; (y-aa) GATA3+GATA2; (bb-dd) Xlim1+Xmsr; (ee-gg) GATA3+Xmsr. Inset, summary of gene expression analysis in the DLP. Dorsal is towards the top in all cases; in a,d,g,j,m,p,s,v,y,bb,ee, anterior is towards the left and lines indicate the levels where sections shown were taken.

or pronephric (GATA3) markers. Panels p-aa and ee-gg were hybridised first with the pronephric marker, GATA3 (turquoise) and secondly (purple) with endothelial (Xfli1, XHex, Xmsr) and blood (SCL, GATA2) markers. The only combination of endothelial and blood markers strong enough to give a clear result was Xfli1 (turquoise) followed by SCL (purple) (panels m-o).

The pronephric genes Xlim1 and GATA3 and the endothelial gene Xfli1 show complete overlap (Fig. 3C, parts a-c,j-l,p-r). Together these genes span the whole of the DLP region dorsally (Fig. 3C, blue and red areas in summary). The expression of the blood genes SCL and GATA2 (Fig. 3C, parts d-f,m-o,s-u,y-aa) along with the endothelial markers XHex and Xmsr (Fig. 3C, g-i,v-x,bb,ee) are restricted to a few cell layers at the dorsoventral end of the DLP region (red area in summary, Fig. 3C). Thus, the only cells expressing all the blood and endothelial genes tested, and therefore the best candidates for a progenitor population, are located at the dorsal extent of the DLP region (red area in summary, Fig. 3C). However, at this stage of their development, these cells are also expressing genes that are later restricted to pronephric duct cells (Xlim1 and GATA3). These two pronephric duct genes are also expressed in cells located more ventrally in the DLP region, in this case without the majority of the blood and endothelial genes (blue area in summary, Fig. 3C). It seems likely, therefore, that these cells will go on to form the pronephric duct. The only blood or endothelial gene expressed in these cells at this stage is Xfli1; however, this expression is not maintained in differentiated pronephric duct cells. The significance of this early Xfli1 expression for pronephric duct development is at present unknown. Temporal gene expression in the zebrafish indicates a similar dorsoventral arrangement of cell fates in the lateral plate mesoderm, with pronephric duct gene expression initially overlapping with blood and endothelial gene expression, with subsequent sorting into adjacent domains (Brown et al., 2000; Gering et al., 1998). Of note in the Xenopus DLP is the absence of Xaml expression. This is an important distinction between the adult/DLP and embryonic/ventral blood and endothelial progenitor populations. Xaml is not expressed in the adult DLP lineage until haematopoietic precursor cells appear 2 days later associated with the dorsoventral aorta (Ciau-Uitz et al., 2000).

### BMP signalling is required for both anterior and posterior VBI formation

Bone morphogenetic protein (BMP) signalling is required for ventral development and its suppression is essential for dorsal development (reviewed by Dale and Jones, 1999). We have shown that the two blood and endothelial progenitor populations described here derive from both ventral and dorsal regions of the early embryo: the anterior ventral population from the dorsal marginal zone (DMZ), and the DLP population from the ventral marginal zone (VMZ) (this study) (Ciau-Uitz et al., 2000). The blood and endothelium of the posterior VBI

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**Table 1. Effects of tBR mRNA injection on gene expression in VBI compartments**

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500 pg tBR mRNA was injected either into the VMZ or DMZ of 4-cell stage embryos and the effects on VBI development analysed by whole mount in situ hybridisation of stage 26 embryos. aVBI, anterior VBI; pVBI, posterior VBI; n, number of embryos analysed. Numbers in remaining columns are percentages.
also derive from the VMZ. To explore its role in the specification of all these blood and endothelial cell populations, BMP signalling was inhibited separately in derivatives of the VMZ and DMZ. Inhibition was achieved by injection of RNA coding for a dominant negative, truncated form of the BMP type I receptor, tBR (Northrop et al., 1995; Suzuki et al., 1994).

Four-cell embryos were injected either dorsally or ventrally in the marginal zone with RNA encoding tBR, allowed to develop to stage 26 equivalent (tailbud) and then examined for blood and endothelial gene expression (Fig. 4 and Table 1). At this stage of development, globin is normally expressed throughout the VBI, displaying the characteristic V-shape at the anterior end (Fig. 4, row 1, Control). When tBR RNA was injected into the VMZ, a partial second embryonic axis was formed as reported previously (Graff et al., 1994; Northrop et al., 1995; Suzuki et al., 1994), and in the primary axis only the V-shaped anterior globin-expressing cells remained (Fig. 4, row 1, VMZ; Table 1, tBR VMZ). Thus, the more posterior cells failed to form blood in the absence of BMP signalling. To confirm that the absence of globin expression was due to the presence of tBR, embryos were co-injected with β-gal RNA. Fig. 5A confirms that when embryos were injected in the VMZ with β-gal RNA alone, the posterior region was labelled but globin expression in the VBI was unaffected. However, when tBR was co-injected along with the lineage tracer, the progeny of the injected VMZ became dorsalised and β-gal was now found in the neural tube and somites of the second axis, with concomitant loss of globin expression from the posterior VBI (Fig. 5B). The cells that now populated the posterior VBI region (arrowheads in Fig. 5B) were derived from cells that had not experienced the block to BMP signalling. Nonetheless these cells were unable to express globin, presumably because they were derived from a position in the embryo that had not been exposed to the correct signalling environment required to set up the blood programme.

When tBR RNA was injected into the DMZ, little alteration to the gross phenotype was observed because this region is already low in BMP signalling. However, the characteristic V-pattern of globin expressing cells in the anterior blood island was lost and, at best, only a more diffuse patch typical of the posterior VBI remained (Fig. 4, row 1, DMZ; Fig. 5C, lower embryo, arrowhead; Table 1, tBR DMZ). Some injected embryos lost globin expression in the posterior VBI completely (Fig. 5C, upper embryo, arrow; Table 1, tBR DMZ), however this effect was less apparent with earlier blood markers and its significance is currently not understood. Inclusion of the β-gal lineage tracer confirmed the presence of the dominant-negative BMP receptor in dorsal and anterior structures including the anterior VBI (Fig. 5C). A reduction in globin production has been reported previously following injection of tBR at the 32-cell stage into both C1 or C4 blastomeres, which are central to the DMZ or VMZ injected here (Kumano et al., 1999). We conclude that both component populations of the VBI require BMP signalling if they are to develop as red blood cells.

In order to determine when in the primitive blood specification pathway BMP signalling is required, we monitored markers for early blood precursors, namely Xaml, SCL and GATA2. Similar results to those seen with globin were obtained with VMZ and DMZ injections, resulting in loss of posterior or anterior expression, respectively (Fig. 4, rows 2-4; Table 1; data not shown for GATA2). Thus, BMP signalling is required at an early stage in the specification of primitive blood. Furthermore, similar results were obtained with markers for endothelial cells, namely Xfli1 and XHex (Fig. 4, rows 5-8; red arrows identify the vitelline veins which link up anteriorly with the endocardium; black arrowheads indicate XHex expression in the liver). Thus, BMP appears to be essential for the specification of both the primitive blood and endothelium from mesoderm. We therefore conclude that
both dorsal and ventral marginal zones fail to produce blood and endothelium in the VBI region when BMP signalling is blocked.

**Xfli1 expression in embryonic/aVBI progenitors is not dependent on BMP**

We have presented evidence to indicate that blood and endothelium of the aVBI derive from a population of progenitors located near the cement gland at neurula stages. The absence of blood or endothelium in tailbud stage embryos injected with tBR in the DMZ predicts that this population of progenitors would be absent at neurula stages. To monitor this, we injected tBR RNA along with the lineage tracer into the DMZ and probed the embryos at stage 18 (neurula) for SCL and Xfli1 expression. When the β-gal lineage tracer was located in the progenitor region posterior to the cement gland, SCL expression was absent or strongly reduced (Fig. 5, compare E with D, 24/25 embryos). By contrast, Xfli1 expression was retained in the absence of BMP signalling, with clear overlap between the purple in situ hybridisation stain and the turquoise lineage tracer (Fig. 5, compare F with G, 33/33 embryos). Thus, Xfli1 expression is turned on in a BMP-independent manner. However, although inhibition of BMP signalling has no effect on Xfli1 expression at stage 18, the normal expression of Xfli1 at stage 26 in the vitelline veins is not observed (Fig. 4, rows 5 and 6, DMZ; Table 1, tBR DMZ).

Overall, these data suggest that, in the absence of BMP signalling, normal progenitors for embryonic blood and endothelium co-expressing SCL and Xfli1 are not formed, and that these cells are obligate precursors to the blood and endothelium of the anterior VBI. BMP is required in this progenitor population to activate SCL expression but not Xfli1.

In a parallel set of experiments, Walters et al. (Walters et al., 2002) concluded that inhibition of BMP signalling in DMZ derivatives has no effect on SCL expression, as measured by northern blot. They therefore interpreted the requirement for BMP to be in the differentiation of erythroid cells rather than the initial formation of haematopoietic tissue. In our hands, SCL expression does not survive the blockade to BMP signalling. Furthermore we monitored the survival of the injected tBR RNA using the β-globin UTRs as tags in an RNase protection assay (Fig. 6). We found that injected RNA remains abundant through gastrulation but during neurula stages undergoes significant turnover, so that by stage 18 it has essentially disappeared. This implies that its effect precedes the appearance of the embryonic blood and endothelial progenitors. The loss of endothelial cells as well as blood at tailbud stages is also consistent with a failure to make these precursors.

**BMP signalling is required for DLP formation**

The DLP blood and endothelial progenitor compartment derives from the VMZ along with the pVBI (Ciau-Uitz et al., 2000). Whereas tBR injected into VMZs eliminated blood and endothelial expression in the pVBI, expression of all these markers in the DLP was unaffected (black arrows in Fig. 4, rows 2-7, VMZ and Table 1, tBR VMZ). However, when we injected a lineage label along with tBR into the VMZ, the label was located in the neural tube and somites, and did not extend more ventrolaterally into the DLP region (Fig. 5B). Thus the progenitor population in the DLP appeared not to have
Distinct programming of the VBI and DLP progenitors

Analysis of gene expression in the two progenitor populations investigated here reveals differences between them in the temporal appearance of the genes tested. Thus, whereas GATA2 appears to be the earliest gene expressed in the ventrolateral marginal zone (VLMZ), we were able to deliver the lineage label and dominant negative receptor RNA by probing for the xenopus α-globin UTR sequences present in the injected RNA. To detect these signals, we used a loading control. Note that at the end of gastrulation (stage 12) large amounts of tBR mRNA are detected but by neurula stages, the signal is all but gone. By early tail bud stage (stage 22), no tBR mRNA is detected at all.

DISCUSSION

Blood and endothelial progenitor populations in Xenopus embryos

The co-development of blood and endothelium was first described from the observation of developing yolk sacs, using the light microscope (Haar and Ackermann, 1971; Sabin, 1920). Since then, gene co-expression and loss-of-function studies have suggested common genetic regulation of these two lineages (Keller, 2001). However, to date a combination of gene co-expression to identify the cells and lineage tracing to determine their fates has not been performed on any organism. The data presented here, together with our previous study (Ciau-Uitz et al., 2000), allow us to conclude that, of the three separate sources of blood and endothelium in the developing Xenopus embryo, at least two, the embryonic/aVBI and the adult/DLP compartments, go through a progenitor state in which they co-express blood and endothelial genes. Whether or not these cells represent haematangioblasts await the labelling of individual cells in the two populations to confirm their bi-potentiality. However, if subsets of cells within the populations are already pre-programmed to become blood or endothelium, we have thus far been unable to detect such heterogeneity as differential gene expression. We have not been able to detect an equivalent progenitor population co-expressing blood and endothelial genes for the posterior VBI. This could be because they are very transitory, receiving the necessary signal combinations only shortly before the signal to differentiate. Although they are likely to receive their BMP signal during gastrulation (see below), and their differentiation signal much later, the BMP signal may not be sufficient for progenitor formation in this region of the embryo and a second signal may be required closer to the time of differentiation. The identity and timing of the additional signals required are currently under study.

Both populations of progenitors can differentiate into endothelium in situ but migrate to different locations before differentiating into haematopoietic cells. Thus, a subpopulation of the embryonic progenitors stay where they are and differentiate into the endothelial cells of the endocardium and vessels in this region, while the remainder of the population migrate posteriorly into the aVBI and give rise to the embryonic blood and the vitelline veins. Similarly, a subpopulation of the adult progenitors in the DLP stay lateral giving rise to the posterior cardinal veins, while the remainder migrate to the midline and form the dorsal aorta and the haematopoietic clusters. Thus, it appears that the micro-environments in which the two populations of progenitors are formed are unsuited to haematopoietic differentiation.

Fig. 6. Turnover of tBR RNA within injected Xenopus embryos. Detection by ribonuclease protection assay of injected tBR mRNA in whole embryos. Embryos at the four-cell stage were radially injected with 250 pg tBR RNA per blastomere. Injected RNA was distinguished from endogenous BMP receptor RNA by probing for the Xenopus α-globin UTR sequences present in the injected RNA. EF1α is the loading control. Note that at the end of gastrulation (stage 12) large amounts of tBR mRNA are detected but by neurula stages, the signal is all but gone. By early tail bud stage (stage 22), no tBR mRNA is detected at all.

experienced an inhibition of BMP signalling. When we injected the tBR and α-gal RNA more laterally into the ventrolateral marginal zone (VLMZ), we were able to deliver the lineage label and dominant negative receptor into the region of the DLP. With this more lateral injection, double axes were still generated (demonstrating that active tBR RNA had spread to the ventral midline) but lineage label was often found in both axes of the embryo instead of solely in the second axis, as seen with VMZ injections. In addition the embryos were usually very foreshortened lacking posterior structures. At stage 28, expression of SCL in the DLP was compromised in all embryos (n=10). Six out of ten embryos lost all signal for SCL in the DLP (Fig. 5H, arrowheads) and pVBI, while retaining expression in the aVBI (Fig. 5H, arrow). In the remaining four embryos, as well as a robust SCL signal in the aVBI, a few SCL+ cells remained in the DLP region. However, when these embryos were sectioned, we found no overlap of SCL signal and lineage label in the DLP region (data not shown), indicating that, as tBR acts cell autonomously, those SCL expressing cells that remained had not experienced a block in BMP signalling. When we probed embryos injected in the VLMZ with tBR and α-gal RNA for expression of the endothelial marker Xfli1, we found no Xfli1 signal overlapping the α-gal signal (23/24 embryos, Fig. 5I, arrowheads). As for SCL, the aVBI acted as a positive control (Fig. 5I, arrow). Similar loss of expression in the DLP was observed for the pronephric duct gene, Xlim1 (Fig. 5J, arrowheads, 24/24 embryos). Whenever the lineage label missed the DLP, expression of all these genes occurred normally (see, for example, Fig. 5K, arrowheads). Thus, we conclude that, as for the embryonic lineage, the blood and endothelial progenitor population in the DLP, along with the associated pronephric duct progenitors, is dependent on BMP signalling for its integrity. However, in contrast to the embryonic population, progenitors in the DLP are dependent on BMP signalling for expression of both Xfli1 and SCL.
mesoderm giving rise to the VBI (Bertwistle et al., 1996), it is preceded by Xfli1 in the DLP (this study). Furthermore, although XHex is expressed before SCL in the precursors of the aVBI (Jones et al., 1999), it comes on at the same time as SCL in the DLP (this study). Finally, Xaml is expressed early in the aVBI precursors (this study), but only in the derivatives of the DLP after they have migrated to the midline and the dorsal aorta has been formed (Ciau-Uitz et al., 2000). Therefore, the temporal cascades of gene expression in the adult/DLP and embryonic/aVBI progenitor populations suggest that distinct programming is taking place.

The separate origins of the adult/DLP and embryonic/VBI progenitors in the embryo (Ciau-Uitz et al., 2000) also raised the possibility that the cells might come under the influence of distinct signalling regimes as they move through the embryo to their sites of differentiation. To test this, we perturbed a signal thought to be important in blood formation: BMP. We found programming differences between the adult and embryonic populations in terms of the dependence of Xfli1 expression on BMP signalling. Thus, while expression of this gene requires BMP signalling in the adult/DLP progenitors, it is activated without BMP in the embryonic/aVBI progenitor population. Its later expression in the endothelial derivatives of the embryonic/aVBI progenitors, however, is not maintained in the absence of BMP. Although the significance of this difference is not currently known, it is intriguing to note that the cloche mutation in zebrafish, which affects both blood and endothelium and has not yet been identified (Stainier et al., 1995), similarly fails to prevent the initial expression of Fli1 while abolishing its later expression (Brown et al., 2000). This similarity raises the possibility that cloche encodes a protein in the BMP signalling pathway. The observation that cloche behaves both cell-autonomously and cell non-autonomously (Stainier et al., 1995) could be consistent with this notion if one takes account of the positive-feedback loop involved in maintenance of BMP expression (Jones et al., 1992). Finally, we have found that the enhancer that drives expression of the mouse Scl gene in blood progenitors and endothelial cells, drives expression of a reporter gene in transgenic Xenopus embryos in adult/DLP progenitors (Gottgens et al., 2002). This enhancer has binding sites for the transcription factors Fli1, GATA2 and Elf1 in multipotential blood progenitors, and all of these sites are required for the activity of the enhancer in the DLP. In adult/DLP progenitors, the loss of Xfli1 expression as a consequence of blocking BMP signalling is therefore a likely explanation for the loss of SCL expression. Such a scenario is supported by the timing of expression of these two genes in the DLP with Xfli1 preceding the appearance of SCL (Fig. 3B). If SCL expression is driven by the same enhancer in embryonic/aVBI progenitors, its loss at neurula stages when BMP signalling is blocked cannot be explained by an effect on Xfli1 whose expression is maintained. However, activity of the enhancer also depends on GATA2 binding, the expression of which in the aVBI progenitors does require BMP signalling (data not shown).

Timing of the BMP requirement and the role of dorsoventral patterning

A number of observations suggest that the BMP requirement for all three populations of blood and endothelial cell precursors is during gastrulation. Firstly, Kumano et al. demonstrated a profound inhibition of globin expression in the VBI as a result of injection of BMP antagonists into the A4 blastomere at the 32 cell stage (Kumano et al., 1999). This blastomere contributes to ventral anterior ectoderm, a rich source of BMP. The gastrulating leading edge mesoderm, which gives rise to the VBI, migrates towards the ventral midline finally contacting the ventral anterior ectoderm at the end of gastrulation (Keller, 1991; Bauer et al., 1994). Second, our studies of the DLP progenitor population show that it develops in close association with precursors of the pronephric duct, which also require BMP signalling. The pronephric duct has been shown to be specified no later than stage 14, which is soon after gastrulation, suggesting that BMP is required earlier (Brennan et al., 1998; Brennan et al., 1999). Finally, our time course on the stability of injected dominant-negative BMP
receptor RNA is consistent with a requirement for BMP during or soon after gastrulation.

We suggest that the dorsoventral gradient of BMP activity thought to exist during gastrulation (Dosch et al., 1997) is important in specifying the different blood and endothelial compartments (Fig. 7A). At the highest level of BMP signalling the posterior VBI is specified; at an intermediate level BMP specifies DLP precursors. Both of these suggestions are consistent with the gradient model and, in the case of the posterior VBI, fit the many experimental results linking blood formation with high BMP (Dale, 1992; Jones et al., 1992; Dosch et al., 1997; Maeno et al., 1994b). However, the specification of the aVBI requires more explanation. These cells derive from the dorsal marginal zone, specifically blastomeres C1 and D1, which also give rise to Spemann’s organiser. The cells of the organiser are characterised by the expression of low levels of BMPs and high levels of BMP antagonists such as chordin and noggin. However, this region contains the first cells to involute during gastrulation (Keller, 1991) and, therefore, at an early stage during gastrulation, they will be proximal to high levels of BMP signalling in the animal cap (Fainsod et al., 1994; Maeno et al., 1994b). We suggest that it is at this stage that C1 and D1 progeny become committed to the blood and endothelial programmes (Fig. 7B).

In addition to this exposure to BMP in the animal cap soon after the start of migration, the initial location of the aVBI precursors in the low BMP organiser territory may also be important for their specification. Three lines of evidence support this. First, Tracey et al. showed that treatment of Xenopus single cell embryos with ultraviolet light, which prevents cortical rotation and establishment of the Spemann organiser, resulted in the loss of the aVBI (Tracey et al., 1998). Second, Walters et al. have shown that the injection of BMP4 RNA into the DMZ can lead to a decrease in globin expression as well as an increase depending on the dose (Walters et al., 2002). Thus, at high BMP4, as found by others (Maeno et al., 1994b; Hemmati-Brivanlou and Thomsen, 1995; Maeno et al., 1996), all the mesoderm, including axial, paraxial and dorsolateral plate was converted to ventral mesoderm (which, in this case, is represented by the pVBI) and therefore globin production was increased. However, when a moderate dose of BMP4 was injected, reflected by an intermediate dorsoanterior index in the resulting embryos, the aVBI was converted to more lateral mesoderm, possibly including DLP, which either does not express globin or does so much later. Finally, the three blood compartments seen in the early Xenopus embryo have possible parallels in the zebrafish in the head, the trunk and the region posterior to the anus, as defined by SCL expression (Gering et al., 1998). In the chordin mutant, which lacks the product of the BMP antagonist, chordin, the anterior haemangioblast compartment is reduced while the posterior compartment is expanded (M. Gering and R. P., unpublished). In addition, overexpression of BMP leads to a complete loss of this anterior population (Liao et al., 2002). Thus, as in Xenopus, the anterior population in zebrafish appears to require conditions of low or zero BMP at some time in its ontogeny.

Conclusions

In conclusion, we have presented combined gene expression and lineage tracing evidence that both adult and the anterior component of embryonic blood in Xenopus embryos derive from populations of progenitors that also give rise to endothelial cells. By targeting a dominant-negative BMP receptor RNA to distinct regions of the embryo that give rise to these progenitor populations, we have shown that BMP is needed for the formation of blood and endothelium in both embryonic and adult compartments. Programming by BMP of these blood and endothelial precursor populations most probably occurs during gastrulation. Expression of the endothelial gene, Xfli1, is not BMP dependent in embryonic progenitors but is dependent on BMP in adult progenitors, while the blood gene, SCL, is dependent on BMP signalling in both compartments. These data identify for the first time differential programming by embryonic signals of the adult and embryonic blood and endothelial lineages.

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