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Common genetic control of haemangioblast and cardiac development in zebrafish

Tessa Peterkin[1], Abigail Gibson[2] and Roger Patient[1,*]

Over the past few years it has become clear that over half of the mammalian heart derives from outside the heart field as originally defined. Such a second heart field, however, has not been described in zebrafish, which could explain its smaller, two-chambered heart. Instead, zebrafish have a population of haemangioblasts, which is absent in mammalian embryos, raising the possibility that these cells represent the evolutionary ancestor of the second heart field. Here, we show for the first time that the genetic programmes of these anterior haemangioblasts and the adjacent heart field are co-regulated, by transcription factors previously associated with heart but not blood or endothelial development. We demonstrate that *gata4*, *gata5* and *gata6* are essential for anterior haemangioblast specification, and for subsequent myelopoiesis, acting as early as *cloche* and upstream of *scl*. The requirement for *gata4, gata5* and *gata6* in myeloid, endothelial and cardiac specification is in the mesoderm, but these factors also control, from within the endoderm and the yolk syncytial layer, the migration of the cardiac precursors as they differentiate. This genetic link between the blood/endothelial and cardiac programmes supports the notion that this haemangioblast population in zebrafish is an evolutionary antecedent of the second heart field, and has implications for the differentiation of haemangioblasts and cardiomyocytes from pluripotent cells, and for the origins of stem cells in the adult heart.

KEY WORDS: Myelopoiesis, Cardiogenesis, GATA factors, Second heart field, Haemangioblasts, Transcriptional regulation, Evolution, Adult stem cells, Zebrafish

INTRODUCTION

Of the several candidates for stem cells in the adult heart, only one, in addition to having the classical stem cell characteristics of multipotentiality, clonality and self renewal, has also been identified and traced in living embryos (Martin-Puig et al., 2008). This cell is identified on the basis of expression of the homeodomain transcription factor islet 1, and in the embryo these cells represent the second heart field. In mammals and the chick, the four-chambered heart is formed from two distinct regions of the embryo: the primary heart field, which gives rise to the left ventricle and contributes to the atria, and the second or anterior heart field, which gives rise to the right ventricle, the outflow tract and also the atria (reviewed by Laugwitz et al., 2008). Zebrafish have only two-chambered hearts and no second heart field has currently been detected. Instead, anterior to the primary heart field, zebrafish have a population of haemangioblasts not detected in chick or mammalian embryos.

The anterior lateral plate mesoderm (ALM) in zebrafish is a source of haematopoietic, endothelial and cardiogenic cells, with the blood and endothelium coming from the most rostral region and cardiac tissue deriving from the adjacent more posterior population (Fig. 1A). The blood/endothelial precursors in the ALM co-express genes that are later expressed in either blood or endothelium and have therefore been referred to as a putative haemangioblast population (Brown et al., 2000; Gering et al., 1998; Thompson et al., 1998). These ALM haemangioblasts have only a transient existence (between 5 and 10 somites), eventually giving rise to myeloid cells (macrophages and neutrophils), head endothelium and endocardium (Herbomel et al., 1999; Hsu et al., 2001; Roman and Weinstein, 2000), whereas the more posterior cardiac precursors differentiate into the muscle of the two-chambered heart (Stainier and Fishman, 1992; Stainier et al., 1993).

Manipulation of anterior haemangioblast regulators suggests that this programme is antagonistic to the cardiac programme within the ALM (Gering et al., 2003; Schoenebeck et al., 2007). Thus, ectopic expression of blood and endothelial master regulators suppresses the cardiac programme, whereas knocking them down generates ectopic cardiomyocytes in the rostral haemangioblast territory. It is tempting to speculate therefore that this latent cardiac potential found in the anterior haemangioblast population may have been recruited by amniotes during evolution, generating the second heart field and a larger, more complex heart.

Although the anterior haemangioblast and cardiac progenitors express predominantly distinct sets of genes, a few are expressed in both territories; for example, *gata4*, *gata5* and *gata6* (Reifers et al., 2000; Reiter et al., 1999; Reiter et al., 2001; Schoenebeck et al., 2007) (this study). Clearly, if the anterior haemangioblast population is the evolutionary precursor of the second heart field, one would expect that they would be under common genetic control prior to the separation of the two programmes. Jointly expressed transcriptional regulators such as *gata4*, *gata5* and *gata6* are therefore candidates for such common genetic control. In vertebrates, GATA factors are traditionally described as belonging to two subfamilies: those predominantly expressed and functioning in haematopoiesis and ectodermal patterning (*gata1*, *gata2* and *gata3*), and those playing a role in cardiac and endodermal formation (*gata4*, *gata5* and *gata6*) (Molkentin, 2000; Patient and McGhee, 2002). In zebrafish, *gata4*, *gata5* and *gata6* have indeed already been shown to be required for normal cardiogenesis and the formation of heart precursors (Holtzinger and Evans, 2007; Peterkin et al., 2003; Peterkin et al., 2007). To determine whether the anterior haemangioblast population is under common genetic control with the heart field, we used morpholinos to knock down *gata4*, *gata5* and *gata6*, and show for

[1]Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK. [2]The Victor Chang Cardiac Research Institute, Level 6, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia.

*Author for correspondence (e-mail: roger.patient@imm.ox.ac.uk)

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the first time that they are crucial for anterior haemangioblast formation and subsequent myelopoiesis. This requirement is within the mesoderm, although we also show that *gata5* and *gata6* are required in the yolk syncytial layer (YSL) and the endoderm for the correct migration of cardiac precursors. The ablation of both cardiac and haemangioblast programmes within the ALM suggests that these GATA factors lie at the top of a genetic cascade that is initially common to both of these two lineages. This is confirmed by the continued expression of *gata4*, *gata5* and *gata6* in *scl* morphants and *cloche* mutants, suggesting that these GATA factors lie upstream of, or parallel to, these well-described blood and endothelial regulatory factors. These data genetically link the anterior haemangioblast and cardiac fields, and are consistent with the former being the evolutionary ancestor of the latter.

MATERIALS AND METHODS

In situ hybridisation of zebrafish embryos

Wild-type and cloche [Clo^{m39}] (Stainier, 2001) zebrafish were bred, maintained, and embryos raised and staged using standard conditions (Westerfield, 1993). In situ hybridisations on zebrafish embryos were carried out as previously described (Jowett, 2001). All RNA probes used were labelled with digoxigenin (DIG) and detection of the antibody–alkaline phosphatase was performed using BM purple (Roche). EST clones for *ets1* and *erg1* were obtained from ImaGenes, clone ID 7051215 and 6966926, respectively. The following forward (5′-ATGATGGATAGCCGG-ATCCTCG-3′) and reverse (5′-GTCCATGTCTACATCCTCTCC-3′) primers were used to amplify *uncx4.1* from cDNA using standard PCR protocols. The PCR fragment was cloned into the pGEM T-Easy vector (Promega) and confirmed by sequencing. To make an in situ hybridisation probe, *uncx4.1* was linearised with *Spe*1 and transcribed with T7. Details of all other probes have been described previously (Patterson et al., 2005; Peterkin et al., 2003; Peterkin et al., 2007).

Morpholino and mRNA injections

The *gata5* and *gata6* antisense morpholinos (mo) were designed and manufactured by Gene Tools and sequences have been previously described (Peterkin et al., 2003; Peterkin et al., 2007). Combinatorial injections were mixed at a ratio of 1:5 *gata6*mo:*gata5*mo and titrated to an appropriate level to avoid non-toxic effects: between 1.25-1.50 ng of *gata6*mo and 6.25-8.50 ng *gata5*mo. The sequences of other morpholinos used have been described previously: *scl* splice mo (Patterson et al., 2005); *pu1* mo (Rhodes et al., 2005); and *casanova* mo (Sakaguchi et al., 2001). *scl*, *pu1* and *cas* morpholinos were titrated to around amounts previously described; final quantities of 6.5 ng, 15 ng and 10 ng, respectively, were used. YSL injections were performed at the 1000-cell stage using a standard fluorescent control morpholino (Gene Tools) as a lineage tracer. *scl* and *lmo2* mRNAs were synthesised and injected as previously described (Gering et al., 2003), *etsrp* (*etv2* – Zebrafish Information Network) mRNA was synthesised and injected as described (Pham et al., 2007).

RESULTS

gata4, *gata5* and *gata6* are required for the formation of the anterior haemangioblast

gata4, *gata5* and *gata6* are expressed in the ALM of zebrafish embryos during early somitogenesis, in the region that gives rise to primitive myeloid cells, head endothelium and endocardium (Herbomel et al., 1999; Reifers et al., 2000; Reiter et al., 1999; Reiter et al., 2001; Schoenebeck et al., 2007) (Fig. 1A, ‘anterior haemangioblasts’). Initially forming bilateral stripes either side of the embryo, the precursors migrate to a position just anterior to the heart cone as they differentiate, and the myeloid derivatives then disperse throughout the whole embryo, expressing markers such as *l-plastin* (*lymphocyte cytosolic plastin 1* – Zebrafish Information Network) and *mpx* (*myeloperoxidase*) in monocytes/macrophages and granulocytes, respectively. To determine whether *gata4*, *gata5* or *gata6* play any role in myeloid development, we used morpholinos to combinatorially knock down *gata5* and *gata6*. We have previously shown that these double morphants are essentially a triple knockdown, as *gata4* is no longer expressed, which was re-confirmed here (see Fig. S1A in the supplementary material) (Peterkin et al., 2007). In *gata5* and *gata6* morphants at 24 hours post-fertilization (hpf), both *l-plastin* and *mpx* were severely downregulated (Fig. 1B). Loss of the transcription factor *ikaros*, associated with less-differentiated cells, was also seen in the head region of morphants (Fig. 1B), suggesting that the defect might be prior to terminal differentiation. Probing earlier for *cmyb* and *ikaros* expression confirmed this, as expression was absent in morphants close to the onset of their expression at 10 somites (Fig. 1C). Expression of the key myeloid regulator *pu1* (*spi1* – Zebrafish Information Network) was also ablated at this time (Fig. 1C). These data show that *gata4*, *gata5* and *gata6* are essential for the specification of myeloid cells during development.

Recent studies have identified both redundant and non-redundant contributions from *gata4*, *gata5* and *gata6* during development (Holtzinger and Evans, 2005; Holtzinger and Evans, 2007; Peterkin et al., 2007). To address this issue in the myeloid population, *gata4*, *gata5* or *gata6* morpholinos were injected individually. The expression of both *l-plastin* and *mpx* was much less severely downregulated in all three individual morphants than in morphants in which all three were lost together (compare Fig. S1B in the supplementary material with Fig. 1B), which suggests that their activities are additive. Therefore, for the rest of the experiments described here, *gata5* and *gata6* morpholinos were co-injected, creating a triple knockdown.

The loss of the earliest myeloid regulators at 10 somites led us to explore the extent to which the entire anterior haemangioblast programme is disrupted. *draculin* and *gata2* are the first blood-associated genes to be expressed in the ALM between 1 and 2 somites (Patterson et al., 2005), and expression of both genes was substantially reduced in *gata5* and *gata6* morphants (Fig. 1D, red arrowheads). Likewise, expression of *scl*, *fli1*, *lmo2* and *etsrp*, which are expressed in and required for haemangioblast formation, were severely downregulated (Gering et al., 1998; Liu et al., 2008; Patterson et al., 2007; Patterson et al., 2005; Sumanas and Lin, 2006) (Fig. 1D). Similar downregulation was observed for *erg1*, *ets1* and *hhex*, whose later expression is in endothelial cells (Liao et al., 2000; Liu and Patient, 2008; Sumanas and Lin, 2006) (Fig. 1D, red arrowheads). By contrast, expression of all of these genes in the posterior lateral mesoderm (PLM), which gives rise to erythroid cells and the major vessels, was unaltered (see Fig. S2 in the supplementary material). Staging of these early embryos was confirmed by counting somite numbers after staining for *uncx4.1* expression (Kawakami et al., 2005) (Fig. 1D, white brackets; data not shown). These data demonstrate a very early role for *gata4*, *gata5* and *gata6* in the specification of anterior haemangioblasts.

Rescue experiments were carried out to validate morpholino specificity. The severe downregulation of *fli1*, *etsrp*, *scl* and *pu1* seen in morphants was rescued by the injection of *gata5* and *gata6* mRNA (Fig. 2). Overexpression studies of GATA factors have proven to be difficult because of their strong phenotypes (Haworth et al., 2008; Weber et al., 2000); however, low levels of *gata5* and *gata6* (25 pg) injected into wild-type embryos produced relatively normal embryos morphologically, and the blood-associated genes *pu1* and *scl* were ectopically expressed (Fig. 2). By contrast, very little if any ectopic or increased expression of vascular genes was observed (Fig. 2). A few ectopic patches of cells expressing *fli1* were detected, whereas *etsrp* expression was seen only within the normal

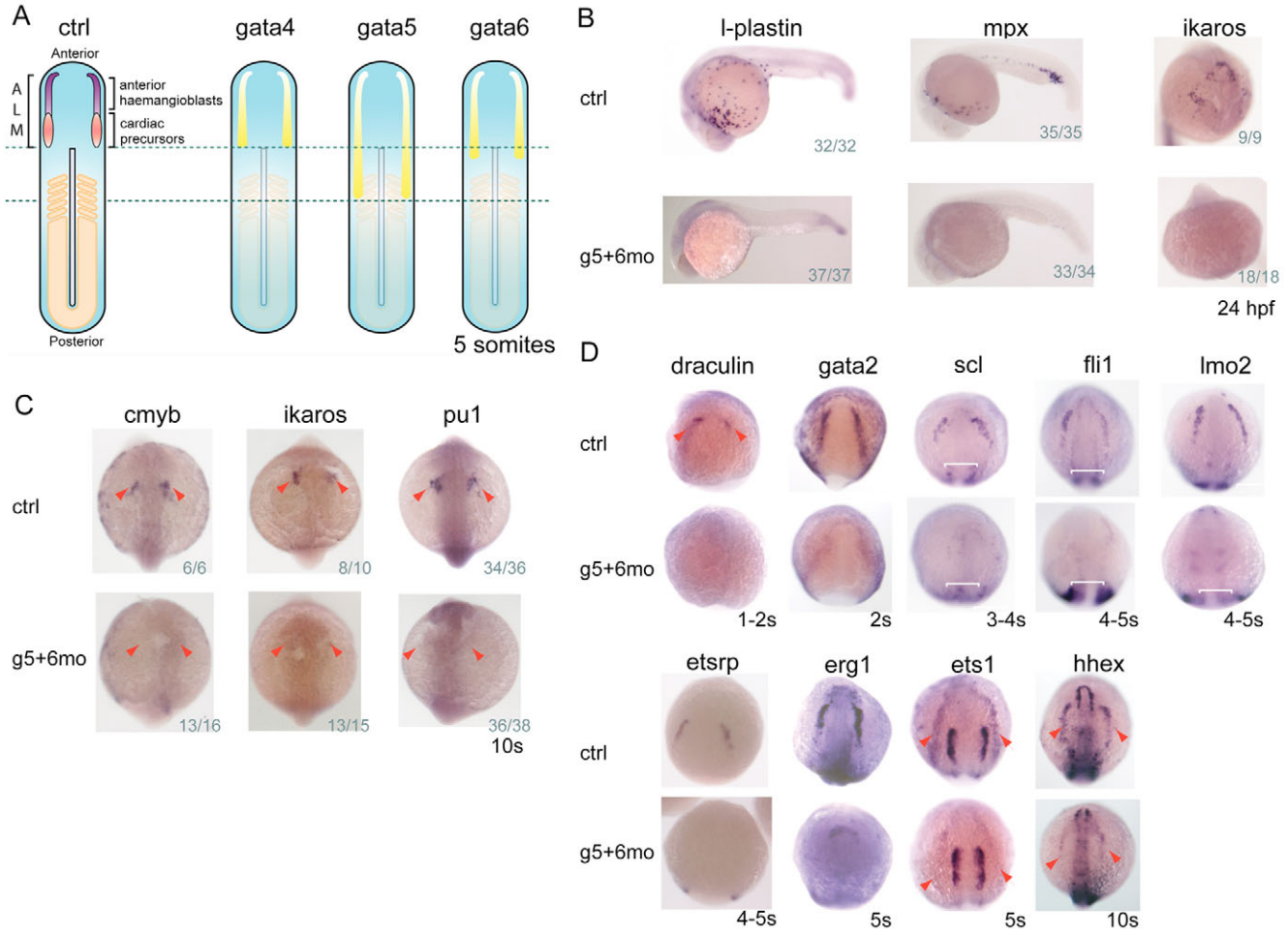

Fig. 1. *gata4*, *gata5* and *gata6* are essential for anterior haemangioblast specification. (**A**) *gata4*, *gata5* and *gata6* are expressed in overlapping yet distinct domains throughout the anterior lateral plate mesoderm (ALM). (**B**) Expression of *l-plastin*, *mpx* and *ikaros*, genes associated with myelopoiesis, was absent in *gata5+gata6* morphants at 24 hpf. (**C**) Analysis of earlier regulatory myeloid gene expression on knock down of *gata5* and *gata6* shows ablation of *cmyb*, *ikaros* and *pu1* at 10 somites (red arrowheads). (B,C) The number of embryos exhibiting this phenotype shown at the bottom right-hand corner of each panel. (**D**) Expression of haemangioblast-associated genes close to the onset of their expression was downregulated in *gata5+gata6* morphants (marked by red arrowheads where necessary). *uncx4.1* was used as a somite marker to stage the embryos (white brackets). *n*=40-100 for each gene analysed, the pictures shown depict >95% of the embryos. Views are: (A) flatmounted, anterior to the top; (B) lateral, anterior to left, except for *ikaros*, which is an anterior view; (C) dorsal, anterior to the top; (D) anterior-dorsal.

bilateral ALM stripes. Thus, although *gata5* and *gata6* are necessary for all haemangioblast gene expression in the ALM, they are sufficient only for myeloid and not vascular gene expression.

gata5 and *gata6* play a migratory role in the YSL

gata5 and *gata6* are expressed in the endoderm and the yolk syncytial layer (YSL), as well as in the ALM, in zebrafish embryos (Kikuchi et al., 2001; Reiter et al., 1999; Rodaway et al., 1999) (A.G., T.P. and R.P., unpublished). The YSL appears to be crucial for migration of cardiac precursors to the midline, with YSL defects giving rise to cardia bifida (Sakaguchi et al., 2006). Cardia bifida is also seen in *gata5* and *gata6* morphants (Peterkin et al., 2007), suggesting that these factors may be acting in the YSL. We therefore wished to determine whether the activity affecting anterior haemangioblast programming is located in the YSL or the ALM. To deplete *gata5* and *gata6* in the YSL, 1000-cell stage embryos were injected into the YSL with *gata5* and *gata6* morpholinos, along with a fluorescent morpholino as a tracer. At this stage of development, the YSL gap junctions are complete and no longer open to the embryo itself (Chen and Kimelman, 2000; Kimmel and Law, 1985). Embryos fluorescent only in the YSL were selected at two different stages as embryos lacking *gata5* and *gata6* in the YSL alone (Fig. 3A, +YSL). Embryos with no fluorescence (–YSL) were collected as injection controls, expressing *gata5* and *gata6* at wild-type levels. To confirm the efficiency of the morpholinos, a batch of embryos was injected at the one-cell stage, giving rise to fluorescence, and therefore *gata5* and *gata6* inhibition, throughout the embryo (+embryo). Cardiac and myeloid gene expression was monitored.

As expected, the levels of expression of the cardiac markers *cmlc2* and *nkx2.5* were normal in the injection controls (–YSL), as were the locations of the expressing cells (Fig. 3A). However, in the embryos in which *gata5* and *gata6* were absent in the YSL alone (+YSL), 100% of the embryos showed cardia bifida, although the levels of expression remained normal (Fig. 3A). In the embryos used as a control for morpholino efficiency (+embryo), *cmlc2* and *nkx2.5* expression was absent, as reported previously (Peterkin et al., 2007). Importantly though, the expression of the myeloid marker *l-plastin* also remained unchanged in + and –YSL embryos, but was ablated

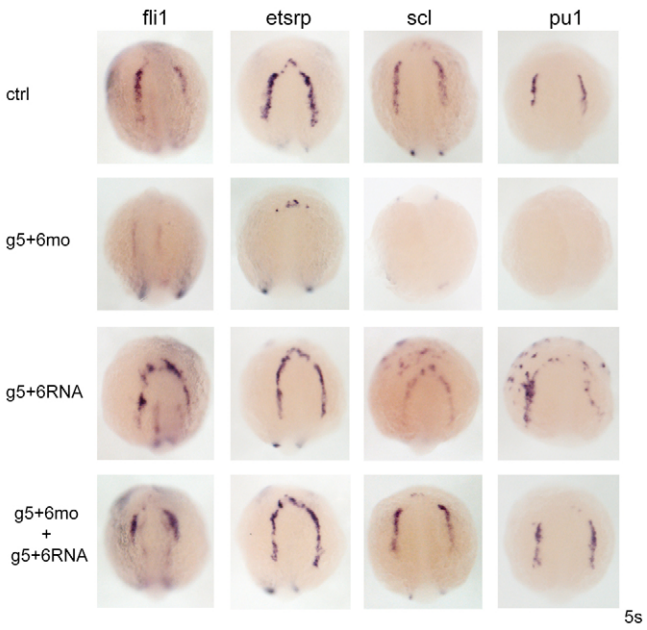

Fig. 2. *gata5* and *gata6* rescue and gain-of-function experiments confirm necessity and identify sufficiency for myelopoiesis. Injection of exogenous *gata5* and *gata6* mRNA (g5+6RNA) into wild-type embryos did not induce *fli1* or *etsrp*, whereas *scl* and *pu1* were ectopically expressed. Rescue of all four genes was seen in *gata5* and *gata6* morphants co-injected with *gata5* and *gata6* mRNA (g5+6mo + g5+6RNA) when compared with *gata5* and *gata6* morphants alone (g5+6mo). Views are anterior-dorsal.

when *gata5* and *gata6* MOs were injected throughout the embryo (+embryo; see Fig. 3A). Thus, *gata5* and *gata6* do not appear to be required in the YSL for myelopoiesis, but rather in the embryo proper. These experiments also show that, whilst expression of *gata5* and *gata6* is required in the YSL for cardiac migration, it is not required there for heart specification or differentiation.

gata5 and *gata6* play a migratory role in the endoderm

In zebrafish, endoderm is not thought to be required for the specification and differentiation of heart precursors, but it is required for the correct migration of cardiac progenitors to the midline (Alexander et al., 1999; Kikuchi et al., 2001; Schier et al., 1997; Trinh and Stainier, 2004). However, its role in myeloid development has not been assessed, and therefore the role of *gata4*, *gata5* and *gata6* in the embryo proper (shown above) could in principle be in the endoderm. In *casanova* (*cas*) mutants, which have no endoderm, *gata4*, *gata5* and *gata6* expression is still observed in the mesoderm (Alexander et al., 1999; Kikuchi et al., 2001), thus the roles of *gata4*, *gata5* and *gata6* in the endoderm and mesoderm should be distinguishable. When wild-type embryos were injected with a *cas* morpholino, endodermal *gata4*, *gata5* and *gata6* expression was lost at 27 hpf, as has been described for *cas* mutants (Alexander et al., 1999) (see Fig. S3A in the supplementary material, red arrowheads), whereas the expression of these GATA factors in the cardiac mesoderm was maintained, albeit in a pattern similar to that seen in cardia bifida, as is seen for *cmlc2* (see Fig. S3A in the supplementary material, black arrowheads). Importantly, expression of *l-plastin* and *mpx* at this time was not altered, suggesting that endoderm, and therefore *gata4*, *gata5* and *gata6* expression within the endoderm, is not required for myelopoiesis at 27 hpf (Fig. 3B).

Expression of *gata4*, *gata5* and *gata6* in the ALM at 5 somites was unaffected in *cas* morphants (see Fig. S3B in the supplementary material). Furthermore, expression of *pu1* at 5 somites and *runx1*, *ikaros* and *cmyb* at 10 somites was also unaffected (Fig. 3C). As a control for morpholino activity (Dickmeis et al., 2001), expression of the endodermal marker *sox17* was lost by 50-90% epiboly (data not shown). We therefore conclude that *gata4*, *gata5* and *gata6* are required in the mesoderm for anterior haemangioblast, as well as cardiac, specification, whereas *gata5* and *gata6* are required in the endoderm and the YSL only for the migration of cardiac precursors.

Epistatic relationships between *gata4*, *gata5* and *gata6* and haemangioblast genes

Having established that the anterior haemangioblast requirement for *gata4*, *gata5* and *gata6* is in the mesoderm itself, we wanted to determine their position in the genetic hierarchy. *pu1* expression is lost in *gata5* and *gata6* morphants but, although this could explain the absence of myelopoiesis, a role for *pu1* in haemangioblast formation or endothelial development has not been established (Rhodes et al., 2005; Su et al., 2007). However, analysis of *pu1* morphants showed that *pu1* is required only for myeloid development and not at all for the endothelial programme (see Fig. S4B,C in the supplementary material). We conclude that the loss of *pu1* in *gata5* and *gata6* morphants (Fig. 1C) and the continued expression of *gata4*, *gata5* and *gata6* in *pu1* morphants (see Fig. S4B in the supplementary material), together with the more widespread defects observed in haemangioblast formation in *gata5* and *gata6* morphants, places *gata5* and *gata6* upstream of *pu1*. We also note that, even though *pu1* was absent in the ALM of *gata5* and *gata6* morphants, ectopic *globin* and *gata1* was never seen there, in contrast to *pu1* morphants (Fig. S4B in the supplementary material; data not shown) (Rhodes et al., 2005). Thus, in the absence of *gata4*, *gata5* and *gata6*, the ALM is unable to form either myeloid or erythroid blood, which is consistent with a position higher up the hierarchy than *pu1*.

scl has been implicated in haemangioblast formation in both the ALM and the PLM of zebrafish embryos (Bussmann et al., 2007; Gering et al., 2003; Patterson et al., 2005). In the ALM, expression of the blood genes *pu1*, *cmyb*, *runx1* and *ikaros* is disrupted, along with expression of the endothelial genes *flt4* and *hhex*, when *scl* is lost, placing it towards the top of the anterior haemangioblast hierarchy (Patterson et al., 2005). To establish the relationship between *scl* and *gata4*, *gata5* and *gata6*, the expression of GATA factors was investigated in *scl* morphants. Embryos were injected with the *scl* morpholinos previously described (Patterson et al., 2005). Continued expression of *gata4*, *gata5* and *gata6* was seen in *scl* morphants from 7 somites (Fig. 4A) to around 15 somites (data not shown). As a control for morpholino functionality, *pu1* was lost in *scl* morphants, as has been shown previously (Patterson et al., 2005) (Fig. 4A). Thus, as *scl* is lost in GATA morphants (Fig. 1D), and *gata4*, *gata5* and *gata6* expression is maintained in *scl* morphants (Fig. 4A), *gata4*, *gata5* and *gata6* emerge as upstream regulators of *scl* in the ALM.

cloche mutants lack vascular and haematopoietic tissues, including myeloid cells (Liao et al., 1997; Rhodes et al., 2005; Thompson et al., 1998), and *gata4*, *gata5* and *gata6* lie upstream of *scl* and *pu1*, both of which can partially rescue myelopoeisis in *cloche* embryos (Liao et al., 1997; Rhodes et al., 2005). Determining the expression of *cloche* has not been possible, because of uncertain identification and low expression of the only candidate (Xiong et al., 2008), so we could not monitor *cloche* expression in *gata5* and *gata6* morphants. We therefore assessed the expression of *gata4*,

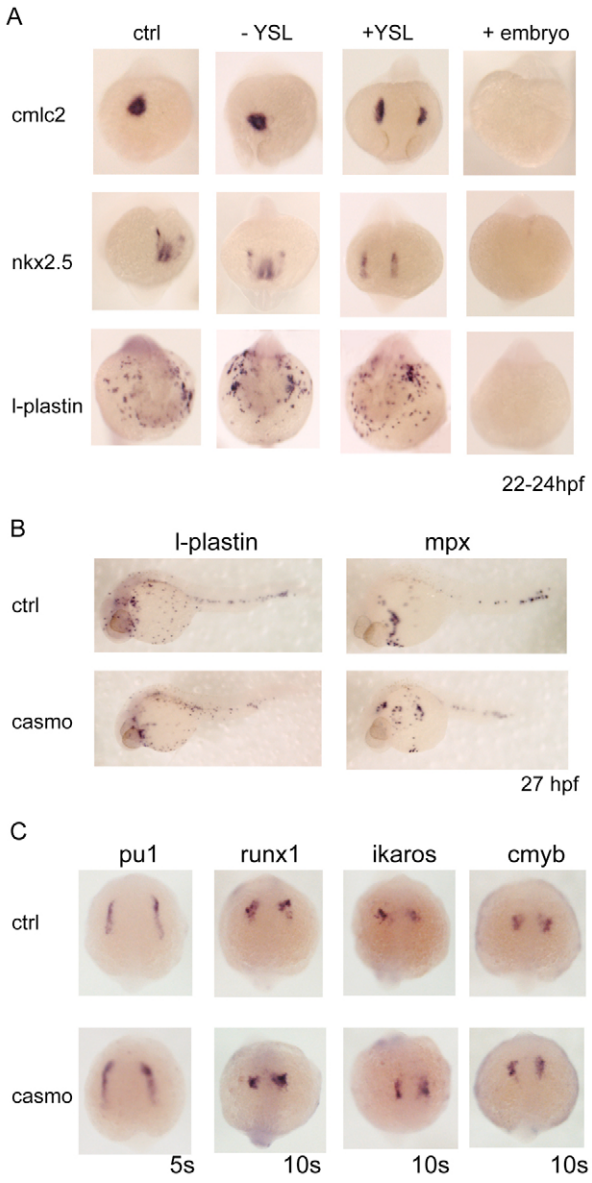

Fig. 3. Depletion of *gata5* and *gata6* from the YSL causes cardia bifida but does not affect myelopoiesis. (**A**) The YSL expression of *gata5* and *gata6* was depleted by injection of *gata5+gata6* morpholinos into the YSL at the 1000-cell stage. To trace the correctly targeted embryos, a fluorescent control morpholino was co-injected. Embryos positive for fluorescence in the YSL alone (+YSL) were selected at several time points and harvested as YSL *gata5+gata6*-depleted embryos. The embryos showing no fluorescence (–YSL) were collected as negative injection controls and should express wild-type levels of *gata5* and *gata6*. To ensure the efficiency of the morpholinos, positive control embryos were injected with *gata5+gata6* morpholinos at the one-cell stage and were fluorescent throughout the embryo (+embryos). To assess heart formation, *cmlc2* and *nkx2.5* expression was analysed. Depletion of *gata5* and *gata6* in the YSL (+YSL) showed normal levels of expression of both *cmlc2* and *nkx2.5*, indicating that specification occurs normally in these embryos. However, cardia bifida was observed in the +YSL embryos, demonstrating that *gata5* and *gata6* are required in the YSL for the correct migration of the cardiac precursors to the midline. By contrast, *l-plastin* expression was the same as in the wild-type embryos, indicating that *gata5* and *gata6* expression in the YSL is dispensable for myelopoiesis. Embryos injected at the one-cell stage (+embryos) showed complete absence of *cmlc2*, *nkx2.5* and *l-plastin* expression, thereby validating morpholino effectiveness. Views are anterior. For each gene and type (±YSL, +embryo) analysed, *n*=28-38, and the images shown depict the findings for >95% of the embryos. (**B**,**C**) Loss of endoderm in *casanova* morphant embryos but no defects in myelopoiesis. To establish whether endoderm plays a role in myelopoiesis, endodermless embryos were created by injection of *casanova* morpholinos. *Casanova* morphants (*cas*mo) were assessed for endoderm formation and myelopoiesis. Myelopoiesis was not affected in *cas* morphants as *l-plastin* and *mpx* remained unaffected (B). Gene expression in the ALM of *cas* morphants was examined at 5 and 10 somites (C). The formation of myeloid precursors occurred as normal in *cas* morphants. Expression of *pu1* at 5 somites, and *runx1*, *ikaros* and *cmyb* at 10 somites, was unaffected in *cas* morphants. Views are anterior-dorsal. For each gene analysed *n*=38–67, and the images shown depict the findings for >95% of the embryos.

gata5 and *gata6* in *cloche* embryos. To control for both the number of somites and to identify *cloche* embryos from their wild-type siblings, triple in situ hybridisation was performed (Fig. 4B,C). *gata4*, *gata5* and *gata6* expression was monitored in the ALM (red arrowheads), and in the same embryos *uncx4.1* staining of early somites was used as a timing control (white brackets), while the loss of *gata1* expression in the PLM was used to identify *cloche* embryos (Stainier et al., 1995). Expression levels of all three GATA factors remained unchanged in *cloche* embryos (Fig. 4B), even though *pu1* expression in the ALM was lost, as shown previously (Lieschke et al., 2002). Thus, *gata4*, *gata5* and *gata6* lie upstream or parallel to *cloche* in the ALM.

To confirm the hierarchical relationships between *gata4*, *gata5* and *gata6* and the other haemangioblast-associated genes, we tried to rescue myeloid and vascular gene expression in *gata5* and *gata6* morphants. Previous studies have shown that co-injection of *scl* and *lmo2* can strongly induce ectopic haemangioblast gene expression, although myeloid gene expression was not expanded and the cells appeared disorganised (Gering et al., 2003). By contrast, *etsrp* overexpression induced ectopic vasculogenesis and myelopoiesis (Sumanas et al., 2008). Overexpression of *scl/lmo2* or *estrp* mRNAs in wild-type embryos behaved as described above (data not shown). However, injection of *scl/lmo2* mRNAs into *gata5* and *gata6* morphants, while still able to strongly induce *fli1* (as in wild-type embryos), was also able to rescue *pu1* expression, albeit in a disorganised fashion (Fig. 5A). By contrast, although *etsrp* was able to induce *fli1* expression in the *gata5* and *gata6* morphants, it could not rescue *pu1* expression (Fig. 5A). Together with the *gata5* and *gata6* overexpression data above, and with recent evidence that *etsrp* is required for the myeloid programme (Liu and Patient, 2008; Sumanas et al., 2008), these data confirm differential roles for *gata5* and *gata6* and *etsrp* in endothelial versus myeloid development (Fig. 5B). *gata5* and *gata6* are necessary and sufficient for at least *pu1* expression, probably via the induction of *scl*, whereas they are necessary but not sufficient for *etsrp* and *fli1* expression, suggesting that an additional factor is required (X in Fig. 5B). The failure of *etsrp* to rescue *pu1* expression while inducing *fli1* expression in *gata5* and *gata6* morphants, suggests that *etsrp* is dependent on *gata5* and *gata6* for its downstream activity in myeloid but not endothelial development. Similarly, the ability of *gata5* and *gata6* to induce other endothelial markers may depend on *etsrp*.

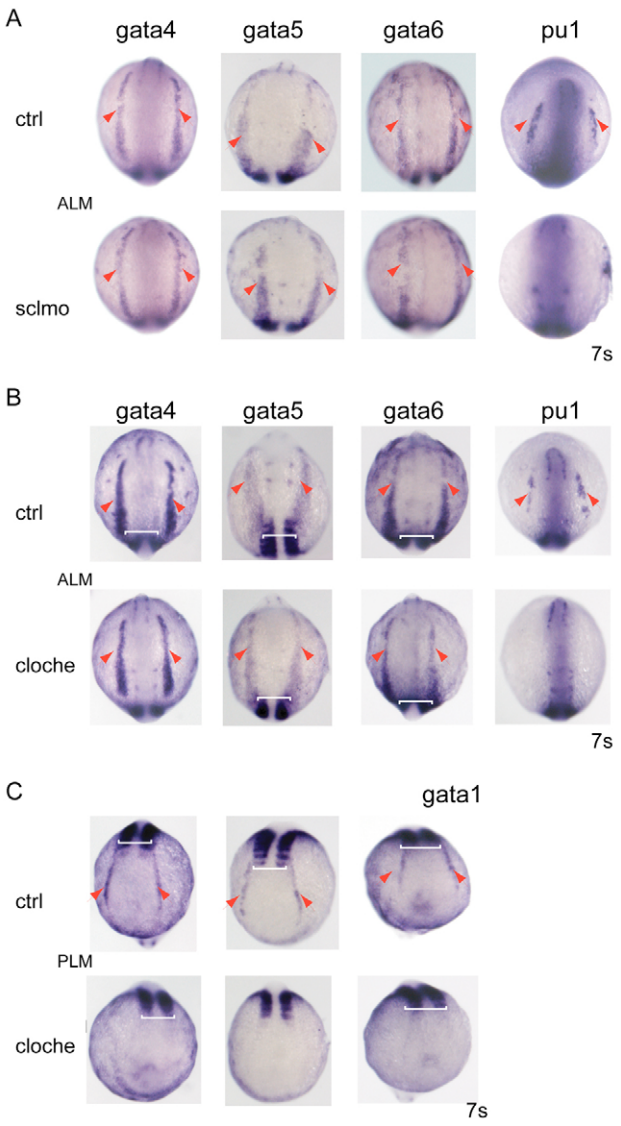

Fig. 4. Initiation of *gata4*, *gata5* and *gata6* expression is independent of *scl* and *cloche*. (**A**) Expression of the GATA factors was analysed in *scl* morphants. *gata4*, *gata5* and *gata6* were expressed as in the wild-type embryos in the ALM (red arrowheads), placing them upstream of *scl*. Loss of *pu1* was used as a control for *scl* morpholino efficacy. (**B**) The expression of *gata4*, *gata5* and *gata6* in the ALM was unchanged (red arrowheads) in *cloche* embryos placing the GATA factors parallel to or upstream of *cloche*. (**C**) *cloche* embryos were identified by the absence of *gata1* expression (red arrowheads) in the PLM. *Pu1* downregulation was an additional control (B). The expression of *uncx4.1* was used as a somitic counter to ensure correct staging of the embryos (white brackets). Views are anterior-dorsal. For each gene analysed, *n*=28-42, and the images shown depict the findings for >95% of the embryos identified as *cloche* and *scl* morphant embryos.

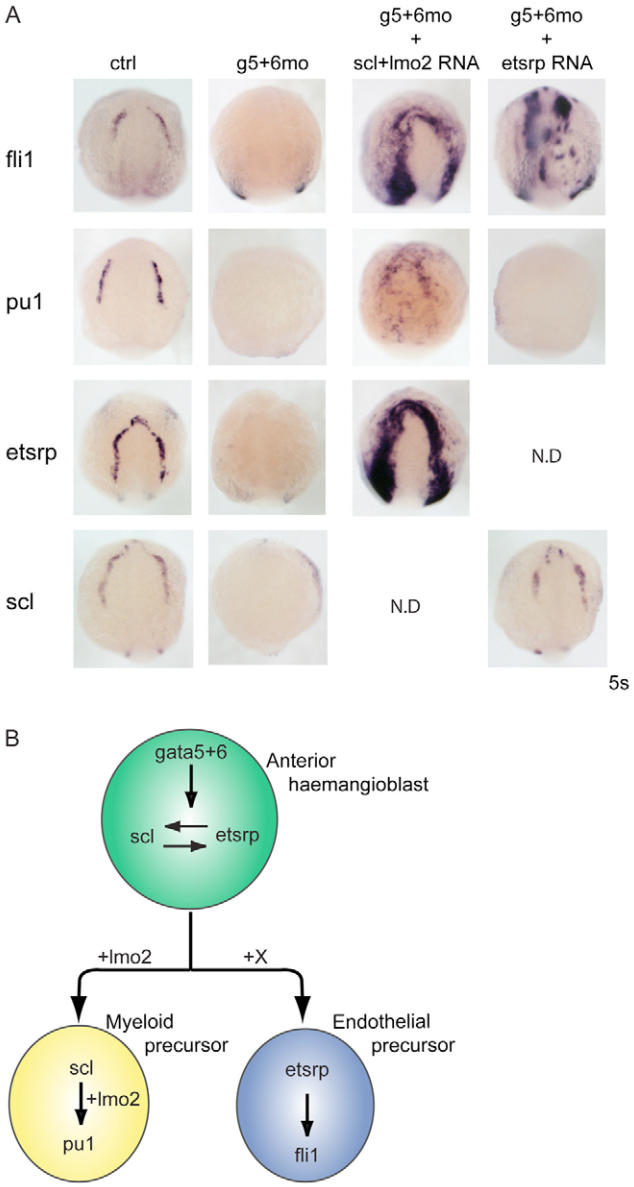

Fig. 5. Interaction of *gata5* and *gata6* with *scl/lmo2* and *etsrp*. (**A**) *Scl/lmo2* mRNA can rescue both blood (*pu1*), and endothelium (*fli1* and *etsrp*) development in *gata5* and *gata6* morphants at the 5 somite stage. By contrast, *etsrp* overexpression can rescue *fli1* but not *pu1* expression in *gata5* and *gata6* morphants. All views are anterior. N.D., not determined. (**B**) Proposed model for *gata5* and *gata6* function within the anterior haemangioblast. *gata5* and *gata6* are required for *etsrp* and *scl* expression in the haemangioblast. *gata5* and *gata6* overexpression can induce *scl* and *pu1* expression, which drives the myeloid lineage, whereas endothelium cannot be induced by *gata5* and *gata6* mRNA (see Fig. 2). Furthermore, the head vasculature eventually recovers (see Fig. S4A in the supplementary material), suggesting the existence of an unknown GATA-independent signal (X).

The fate of *gata4*-, *gata5*- and *gata6*-depleted mesoderm

The data presented here and in previous reports show that *gata5* and *gata6* are required for the specification of the cardiac and haemangioblast programmes in the ALM (Peterkin et al., 2003; Peterkin et al., 2007; Reiter et al., 1999). What happens to these cells in the absence of *gata4*, *gata5* and *gata6*? We showed previously that expression of *nkx2.7* is unaffected in the ALM at 5 somites when haemangioblast- and cardiac-associated genes are already lost in *gata5* and *gata6* morphants, and that no increase in apoptosis was observed at 10 somites (Peterkin et al., 2007). From these data we can conclude that mesodermal cells are still present and have correctly migrated during gastrulation, but that they are unable to differentiate into either the cardiac or haemangioblast lineages.

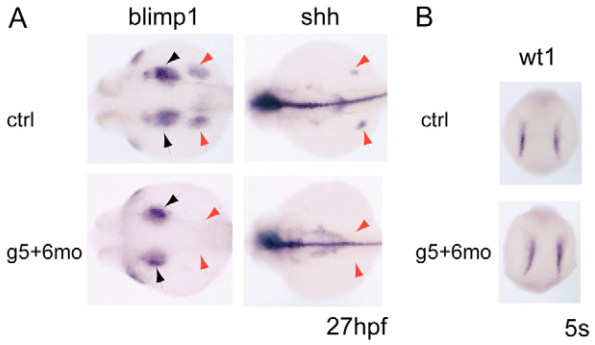

Fig. 6. Fate of ALM cells in *gata5* and *gata6* morphants. (**A**) Fin buds are lost in *gata5* and *gata6* morphants, as seen by the loss of *blimp1* and *shh* expression (red arrows). Pharyngeal endoderm is slightly reduced in morphants (*blimp1*, black arrows) compared with wild type. (**B**) Pronephric mesoderm (*wt1* expression) remains unchanged in *gata5* and *gata6* morphants at 5 somites.

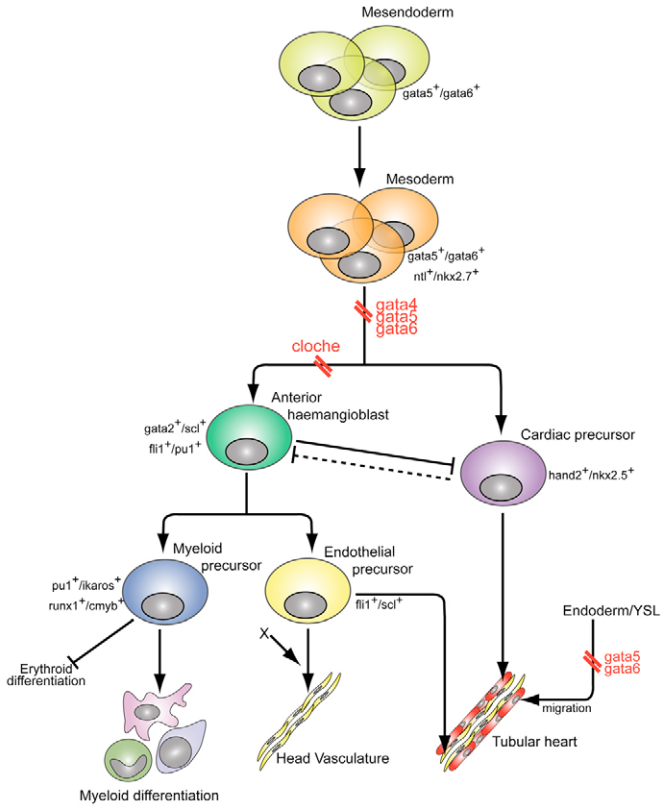

Fig. 7. Cellular hierarchy of anterior haemangioblast and heart formation. *gata4*, *gata5* and *gata6* are required for the specification of the anterior haemangioblast and cardiac precursors from the anterior lateral plate mesoderm (double red break). *gata4*, *gata5* and *gata6* lie at the apex of the hierarchy, above *cloche* (double red break) and *scl*. Previous studies show that loss of the haemangioblast programme causes the expansion of cardiac progenitors (Schoenebeck et al., 2007), suggesting an antagonistic relationship between these two programmes (barred solid and dashed lines). Whether the cardiac precursors can antagonise the anterior haemangioblast population has yet to be demonstrated (dashed line). Data presented here also show a requirement for *gata5* and *gata6* in the endoderm and the YSL for the correct migration of cardiac precursors towards the midline.

Additional mesodermal markers expressed in the ALM in the absence of the haemangioblast and cardiac programmes are not currently available, so we monitored adjacent tissues to determine whether they are expanded in the absence of *gata5* and *gata6*. Fin buds are one candidate; however, they are dependent on *tbx5* (Ahn et al., 2002; Garrity et al., 2002) and *tbx5* is absent in *gata5* and *gata6* morphants at 10 somites (Peterkin et al., 2007). Consistent with this, a loss of fin buds was evident at 27 hpf, as revealed by the loss of *blimp* and *shh* expression (Fig. 6A, red arrows). *blimp* expression in the pharyngeal endoderm was slightly reduced but still present (Fig. 6A, black arrows). *wt1* is expressed in the pronephros just posterior to the heart field (Drummond et al., 1998; Serluca and Fishman, 2001), and this expression was unaffected in *gata5* and *gata6* morphants (Fig. 6B). Thus, in the absence of *gata5* and *gata6*, the haemangioblast, cardiac and fin bud programmes are not induced, but adjacent tissues are not expanded and mesodermal cells expressing *nkx2.7* are still present in the ALM.

What happens to the *nkx2.7*-expressing cells? Expression of myeloid genes such as *runx1*, *ikaros*, *draculin*, *l-plastin* and *mpx* in ALM-derived cells was never seen in *gata5* and *gata6* morphants (Fig. 1; data not shown), whereas endothelial differentiation appeared to have recovered completely by 22 hpf (see Fig. S4A in the supplementary material). This recovery is consistent with previous data (Holtzinger and Evans, 2007), which have shown that expression of *lmo2* appeared normal in *gata5* and *gata6* morphants around 12 somites, a time at which we saw recovery of expression of several endothelial genes, including *lmo2*. Thus, it appears that some of the ALM cells in morphants are still able to contribute to head vasculature.

DISCUSSION

gata4, *gata5* and *gata6* lie at the hierarchical apex of blood and cardiac specification

Data presented here demonstrate for the first time a crucial role for *gata4*, *gata5* and *gata6* in haemangioblast as well as cardiac precursor specification. Although Gata4 and Gata6 have been detected in mouse embryos in and around the blood islands and the allantois, another tissue associated with blood and endothelial development, there has hitherto been no evidence for a cell-autonomous role (Bielinska et al., 1996; Caprioli et al., 2001; Dumon et al., 2006; Nemer and Nemer, 2003). *Gata5* expression has been observed to increase in embryonic stem (ES) cells differentiated towards a haematopoietic fate, but no functional consequences have been demonstrated (Baird et al., 2001). Here, we show for the first time that expression of these 'cardiac' GATA factors is required in the lateral mesoderm for the development of myeloid lineages and the initial programming of their associated endothelial cells.

The presence of *gata4*, *gata5* and *gata6* in the endoderm and YSL, as well as in the cardiac and haemangioblastic mesoderm, raised issues concerning the cell autonomy of the role described here. We demonstrate that *gata5* and *gata6* are indeed crucial in both the YSL and the endoderm for migration of cardiac precursors to the midline, but that they are dispensable there for the specification of both the heart tissue and the haemangioblasts. Thus, the requirement for the GATA factors in specification must reside within the mesoderm. Precedent for inductive interactions between endoderm and blood/endothelial programming comes from experiments performed in mouse and chick (Belaoussoff et al., 1998; Bielinska et al., 1996; Kessel and Fabian, 1987). By contrast, we found that loss of endoderm in *casanova* morphants, including loss of *gata4*,

gata5 and *gata6* expression there, had no effect on haemangioblast specification or myelopoiesis. Differences between zebrafish and mouse/chick may in part reflect the different origins of the endoderm: whereas in chick and mouse, yolk sac haematopoiesis and vasculogenesis occur adjacent to the visceral endoderm, in zebrafish the ALM is adjacent to the definitive endoderm.

The mutated gene in the zebrafish *cloche* mutant is thought to be close to the hierarchical apex of blood and endothelial development (Liao et al., 1997; Rhodes et al., 2005; Thompson et al., 1998). Consistent with this notion, the mouse homologue of *lycat*, a gene cloned from the *cloche* genetic interval, is essential for blood and endothelial specification in ES cells (Wang et al., 2007). We have now shown that *gata4*, *gata5* and *gata6* are required for both haemangioblast and cardiac specification, and that expression of *gata4*, *gata5* and *gata6* is unaffected in *cloche* mutants, placing these GATA genes not only upstream of or parallel to *cloche*/*lycat* in the haemangioblast lineage, but also at the apex of the genetic hierarchy common to both the cardiac and haemangioblast programmes (Fig. 7). Based on their early expression patterns (Rodaway and Patient, 2001), we hypothesise that *gata5* and *gata6* are required very early in the mesendoderm, allowing it to respond to both blood- and cardiac-inducing signals.

Although the requirement for *gata4*, *gata5* and *gata6* for blood development from the anterior haemangioblast is absolute, there appears to be a GATA-independent pathway that is able to rescue endothelial development after GATA expression has ceased. Thus, whereas expression of the myeloid genes in the anterior (*pu1*, *runx1*, *cmyb*, *l-plastin* and *mpx*) was never seen in *gata5* and *gata6* morphants, expression of genes associated with endothelial development (such as *fli1*, *etsrp1*, *ets1* and *hhex*), along with haemangioblast genes (*scl* and *lmo2*), in their later head endothelial mode, began to recover from around 12 somites and appeared normal by 15 somites, resulting in an apparently normal circulatory system (see Fig. S4A in the supplementary material; data not shown). Thus, it appears that a recovery pathway is available for endothelial development from the ALM but not for blood (Fig. 5B, labelled X). As *cloche* embryos show a severe downregulation of endothelium throughout development, it is likely that the recovery of head endothelium in GATA morphants is *cloche* dependent. Thus, even though *gata5* and *gata6* are required together with *cloche* for the initiation of the haemangioblast programme, they are apparently not required for maintenance of the endothelial programme in the haemangioblast derivatives.

gata4, *gata5* and *gata6* and the origins of the second heart field and cardiac stem cells

Attractive candidate stem cells in the adult mouse heart are the islet 1-positive population also found in developing embryos (reviewed by Laugwitz et al., 2008). These cells constitute the second heart field in the mammalian embryo. Although the absence of a second heart field in zebrafish embryos may explain the smaller two-chambered heart produced, the presence of a haemangioblast population anterior to the primary heart field, and its absence in mouse embryos, raises the possibility that this haemangioblast population represents the evolutionary precursor of the mammalian second heart field. Consistent with such a notion, we have found that the two populations do indeed have common genetic control, depending absolutely in both cases on *gata4*, *gata5* and *gata6*. Interestingly, using the marker *islet-1*, a second heart field has recently been reported for the amphibian *Xenopus laevis* (Brade et al., 2007), which has a three-chambered heart and an anterior haemangioblast population (Walmsley et al., 2002), raising the possibility that Amphibia represent an intermediate evolutionary state between fish and amniotes.

Recently, cardiac gene expression has been detected in the anterior haemangioblast population in *cloche* embryos and in *scl*/*etsrp* morphants, suggesting that, normally, the blood/endothelial programme there might be responsible for the suppression of the cardiac programme (Schoenebeck et al., 2007). Consistent with this, overexpression of *scl*, with either *etsrp* or *lmo2*, ablates heart formation (Gering et al., 2003; Schoenebeck et al., 2007). These observations suggest that the blood and cardiac programmes in the ALM are antagonistic. It will be interesting to determine how this antagonism was resolved in favour of the cardiac programme during evolution.

Identifying and characterising stem cells in the adult heart is likely to have implications for treatment of heart disease. Islet 1-positive cells have several of the necessary credentials and are thought to derive from the second heart field. Clearly a better understanding of their genetic programme will facilitate their future manipulation and also their derivation from pluripotent cells. Consistent with the common genetic programme indicated here, cardiomyocytes have recently been obtained from ES cell derivatives expressing the VEGF receptor, *flk-1*, which is classically associated with haemangioblast development (Kattman et al., 2006). Interestingly, haemangioblasts differentiate from ES cells before cardiomyocytes and in less time than it takes to make haemangioblasts in a mouse embryo (Huber et al., 2004). In zebrafish and more obviously *Xenopus* embryos, the anterior haemangioblast programme develops earlier than the posterior blood and endothelial programme (Walmsley et al., 2008), raising the intriguing possibility that the haemangioblasts derived from mouse ES cells are expressing the ancestral programme.

Taken together, our work identifies a close genetic relationship between anterior haemangioblasts and cardiac precursors in zebrafish. We reveal a co-dependence of these populations on *gata4*, *gata5* and *gata6*, placing these genes at the apex of the genetic regulatory hierarchy specifying these two anterior lateral mesoderm derivatives. This common genetic control is consistent with the postulated conversion of one to the other during evolution.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/9/1465/DC1

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