Fgf differentially controls cross-antagonism between cardiac and haemangioblast regulators
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SUMMARY
Fibroblast growth factor (Fgf) has been implicated in the control of heart size during development, although whether this is by controlling cell fate, survival or proliferation has not been clear. Here, we show that Fgf, without affecting survival or proliferation, acts during gastrulation to drive cardiac fate and restrict anterior haemangioblast fate in zebrafish embryos. The haemangioblast programme was thought to be activated before the cardiac programme and is repressive towards it, suggesting that activation by Fgf of the cardiac programme might be via suppression of the haemangioblast programme. However, we show that the cardiac regulator nkx2.5 can also repress the haemangioblast programme and, furthermore, that cardiac specification still requires Fgf signalling even when haemangioblast regulators are independently suppressed. We further show that nkx2.5 and the cloche candidate gene lycat are expressed during gastrulation and regulated by Fgf, and that nkx2.5 overexpression, together with loss of the lycat targets etsrp and scl can stably induce expansion of the heart. We conclude that Fgf controls cardiac and haemangioblast fates by the simultaneous regulation of haemangioblast and cardiac regulators. We propose that elevation of Fgf signalling in the anterior haemangioblast territory could have led to its recruitment into the heart field during evolution, increasing the size of the heart.

KEY WORDS: Fgf, Nkx2.5, Adult stem cells, Haemangioblast, Cross-antagonism, Reprogramming, Zebrafish

INTRODUCTION
The heart has grown in size and capacity during vertebrate evolution, reflected in the two-chambered heart in zebrafish and the four-chambered heart of amniotes, including humans (Simoes-Costa et al., 2005). One possible explanation for this expansion is the contribution of the second heart field (SHF), which is distinct from the first heart field and contributes two or more chambers later in development (Vincent and Buckingham, 2010). However, two phases of cell addition to the zebrafish heart have also very recently been observed, making this explanation unlikely, even though the consequences of knocking down the SHF marker islet1 are minimal for heart development compared with those seen in mice (de Pater et al., 2009; Hami et al. 2011; Zhou et al., 2011). A potential source of cells for heart expansion found in the zebrafish adjacent to the heart field, is a population of blood and endothelial precursors, or haemangioblasts, that are not found in amniotes (see Fig. 1A), raising the possibility that these cells have been recruited into the heart field during evolution. Consistent with this idea, gain- and loss-of-function experiments with blood and endothelial regulators have indicated that these cells possess latent cardiac potential that is held in check by the haemangioblast programme (Gering et al., 2003; Schoenebeck et al., 2007). Furthermore, the anterior haemangioblast and cardiac precursors share genetic control, both requiring gata4, gata5 and gata6 function (Peterkin et al., 2009).

The signals that determine cardiac versus blood/endothelial fates in the anterior lateral plate mesoderm (ALPM) are currently unknown. A role for fibroblast growth factor (Fgf) signalling in heart development has been demonstrated but whether it controls cell fate, survival or proliferation is unknown (for a review, see Zaffran and Frasch, 2002). In zebrafish, the fgf8 mutant acerebellar (ace) exhibits a strong reduction in expression of the heart precursor marker nkx2.5 during early somitogenesis, although some recovery is seen later in development resulting in only a modest reduction in heart size (Reifers et al., 2000). Inhibition of Fgf signalling using a pan Fgf receptor inhibitor, SU5402, showed a more substantial defect suggesting that additional Fgfs might be involved. More recent data supports a reiterative role for Fgf signalling, showing successive requirements, initially for regulation of heart size and chamber proportionality, and then for ventricular maintenance (Marques et al., 2008). Data on the role played by Fgf signalling in blood and endothelial development is somewhat contradictory. In chick and Xenopus, Fgf has been shown to inhibit erythroid differentiation, either in favour of proliferation or of endothelial development (Iraha et al., 2002; Nakazawa et al., 2006; Walmsley et al., 2008), whereas in zebrafish, fgf21 has been shown to be required for erythroid differentiation (Yamauchi et al., 2006). Furthermore, although Fgf has been shown to be essential for the formation from mouse embryonic stem cells of the haemangioblast, it has also been shown to be inhibitory for the subsequent differentiation of these cells into either blood or endothelium (Faloon et al., 2000; Yamada et al., 1994).

In this paper, we compare the effects of Fgf signalling on anterior haemangioblast and heart development in the zebrafish. We find that the loss of cardiac tissue seen when Fgf signalling is inhibited is accompanied by an increase in blood and endothelium, and that this reflects a stable change of fate rather than an effect on survival or proliferation. Individual and combinatorial depletion of Fgf ligands showed that fgf3 and fgf8 are the genes responsible. Temporal inhibition of Fgf signalling demonstrates that this role in
distinguishing these two cell fates occurs during gastrulation. Because the expression of haemangioblast regulators was affected prior to the known onset of cardiac regulator expression, we wondered whether induction of the heart programme by Fgf was via repression of the anterior haemangioblast programme. However, we also found that ectopic expression of the cardiac regulator ntx2.5 inhibited the haemangioblast programme, indicating that the antagonism between these two programmes is mutual. Furthermore, by independently suppressing the haemangioblast programme, we showed that Fgf is still required to drive the cardiac programme. Of the known haemangioblast and cardiac regulators, we found that lycat, a cloche candidate gene (Xiong et al., 2008), and ntx2.5 are expressed during gastrulation in an Fgf-dependent manner. Furthermore, Fgf-independent repression of haemangioblast regulators together with overexpression of ntx2.5 led to a bigger heart, with both atrial and ventricular gene expression stably upregulated. Overall, these observations indicate that the ratio of cardiac to blood/endothelial cells in the developing embryo is determined in part by the magnitude of Fgf signalling, and that an elevation of Fgf signalling could have been recruited into the heart field (HF) during evolution.

MATERIALS AND METHODS

Zebrafish strains

Wild-type (WT) and transgenic kdr::GFP and cmlc2::GFP (Beis et al., 2005; Huang et al., 2003) embryos and adult fish were raised and maintained as described (Westerfield, 1993).

SU5402 treatments

WT and scl+etsrp morphant embryos were treated with 5 and 10 μM SU5402 (Mohammadi et al., 1997) (Calbiochem) from different time points, for different periods of time. Embryos were incubated in the dark at 28.5°C and fixed at appropriate stages. Control embryos were treated with an equal volume of DMSO added to fish water.

Antisense morpholino oligonucleotide injections

WT embryos were injected at the 1- to 8-cell stage with morpholinos (MOs) obtained from GeneTools (Philomath, OR, USA). Fgf3 (Phillips et al., 2001), fgf9 (Furthauer et al., 2001), fgf24 (Fischer et al., 2003), scl (Patterson et al., 2005), etsrp (Sumanas and Lin, 2006) and ntx2.5 (Sultana et al., 2008) MOs were injected at published levels and embryos were fixed at described stages.

Ntkx2.5-GFP plasmid generation and RNA injection

Ntkx2.5 PCR fragments were generated via Superscript III One-Step RT-PCR System (Invitrogen), using total RNA extracted from 5-somite embryos, with the following primers (5'-3'): ntx2.5-gateway-Forward: GGGGACAAGTTGTGACAAGGCTCCATGGCAGATGGTTCTCTTAGCC; ntx2.5-gateway-Reverse: GGGGACACTTTGTACAAAGAAGCTGGTGCCAAAGCTCTGATCATG. Gateway cloning technology (Invitrogen) generated an ntx2.5 entry vector in pDONR221 backbone, which was recombined with pCSGF2 (Gering et al., 1998) to create an ntx2.5-GFP plasmid, in which the partial 5' UTR and full coding sequence of the gene, minus the stop codon, were placed immediately upstream of the coding sequence of GFP. Full length, capped RNA (50 pg) was injected into 1- to 2-cell stage zebrafish embryos, which were raised at 28.5°C and fixed in 4% paraformaldehyde.

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation on zebrafish embryos was carried out as previously described (Jowett and Yan, 1996). Antisense RNA probes were transcribed from linearised templates in the presence of digoxigenin (DIG) or fluorescein labeled nucleotides (Roche). DIG and fluorescein antibodies were detected using BM-Purple (Roche) or Fast Red (Sigma), respectively.

TUNEL and antibody assays

For detection of DNA strand breaks in apoptotic cells, TUNEL staining of zebrafish embryos was performed with the Fluorescein In Situ Cell Death Detection Kit (Roche) and detected using BM-Purple (Roche). Immunohistochemistry for detection of cell proliferation was performed as previously described (Mably et al., 2003), using the rabbit antibody against phospho-histone H3 (Ser28) (Upstate) at a 1:500 dilution. The secondary goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Invitrogen) was used at a working dilution of 1:500. DAPI was used for nuclear counterstaining. Immunohistochemistry using MF20 (Developmental Studies Hybridoma Bank) (1:200) followed an Alexa 555 goat antimouse monoclonal IgG2b (Invitrogen) (1:500) was performed as previously described (Reifers et al., 2000).

Quantitative PCR

Total RNA was isolated with the RNAeasy Micro Kit (QIAGEN). Quantitative PCR (qPCR) was performed with Taqman [at 36 hours post-fertilisation (hpf)] or Sybr Green [50% epiboly, 7 somites and 48 hpf] (Applied Biosystems). Data were collected with the ABI-PRISM 7000 or 7500 Sequence Detection System. Gapped was used as an internal control, and the relative abundance for each sample was computed by the comparative method (ΔΔCt). Statistical analysis was by the two-sample equal variance t-test. Error bars indicate the s.e.m. Four and three biological replicates for each pool of 20 dissected heart-regions of 36 hpf and 48 hpf embryos were used, respectively, and three biological replicates for each pool of 20 whole 50% epiboly and 7-somite embryos. The dissections removed the tail vasculature and the anterior of the head including the eyes. See Table S1 in the supplemental material for a list of the primers used. Previously published primers used are described (Gomez et al., 2009; Rikin and Evans, 2010).

Imaging

Images were obtained on a Nikon SMZ 1500 zoom stereomicroscope using a Nikon DXM 1200 digital camera (Nikon, UK). GFP embryos were visualised using a Leica MZ FLIII fluorescence stereomicroscope (Leica Microsystems, UK) and photographed with a Hamamatsu ORCA-ER camera driven by Simple PCI version 5.1.0.0110 software (Compix, USA). Confocal images were obtained using a Zeiss LSM 510 META confocal laser microscope, and 3D projections were generated using Zeiss LSM software (Carl Zeiss). All images were processed with Adobe Photoshop CS3 software (Adobe Systems, San Jose, CA, USA).

RESULTS

Inhibition of Fgf signalling induces blood gene expression in the heart field

Anterior haemangioblast gene expression is normally restricted to cells located immediately adjacent to the HF (Fig. 1A), and gain-and loss-of-function studies have revealed a repressive role for this programme towards the cardiac programme (Gering et al., 2003; Schoenebeck et al., 2007). To determine the effect of Fgf signalling on the haemangioblast programme, zebrafish embryos were treated with the pan Fgf receptor inhibitor SU5402 (Mohammadi et al., 1997) from 3 hpf (hours post-fertilisation) at 5 and 10 μM concentrations to give rise to a mild to strong reduction of Fgf signalling, but not elimination, which would prevent successful completion of gastrulation (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). Confirmation that Fgf signalling had been significantly reduced was given by reduced expression of pea3 (see Fig. S1A in the supplementary material), an Fgf signalling pathway target gene (Marques et al., 2008; Reifers et al., 2000; Roehl and Nusslein-Volhard, 2001). In addition, and in accordance with reported Fgf roles, embryos exposed to SU5402 had a short body axis, reduced posterior mesoderm and hindbrain defects, including loss of rhombomere 5 (Griffin and Kimelman, 2003).
To confirm the effect of lost Fgf signalling on the heart field and extend it to additional markers, cardiac gene expression was assayed at the 7-somite stage, just after the onset of expression of nkx2.5, which marks the cardiac precursor pool (Chen and Fishman, 1996). \textit{mef2c} is present in the pre-myocardial mesodermal cells (Ticho et al., 1996) and \textit{hand2} has been shown to be important in setting up the HF (Schoenebeck et al., 2007; Yelon et al., 2000). Expression of all three transcription factors was severely downregulated in the cardiac field of SU5402-treated embryos (Fig. 1B, brackets). There were still some \textit{hand2}+ \textit{nkx2.5}– cells remaining in the cardiac mesoderm of inhibitor-treated embryos. The reduction of \textit{nkx2.5} expression and lack of significant effect on \textit{gata6} were confirmed by qPCR (see Fig. S1B in the supplementary material).

We therefore conclude that the conditions of Fgf inhibition that we have used recapitulate the negative effects on \textit{nkx2.5} expression reported previously (Reifers et al., 2000), and that the concomitant reductions we observed for \textit{mef2c} and \textit{hand2} indicate a substantial impact on the entire cardiac programme.

To determine the effect of lost Fgf signalling on the anterior haemangioblast programme, we monitored the expression of a range of genes associated with blood and endothelial development at the 7-somite stage (Bussmann et al., 2007; Fouquet et al., 1997; Gering et al., 1998; Kalev-Zylinska et al., 2002; Liu et al., 2008; Sumanas et al., 2008). In all cases we found that their expression expanded into the presumptive cardiac field (Fig. 1C, brackets). Expression was also upregulated within the haemangioblast field itself, indicating that Fgf normally limits expression here as well. The overall increase in expression was confirmed by qPCR for \textit{pu.1} (\textit{spi1} – Zebrafish Information Network) (see Fig. S1B in the supplementary material). We therefore conclude that Fgf signalling restricts haemangioblast gene expression in a graded fashion within the cardiac and the haemangioblast fields. We note that...
haemangioblast gene expression expanded only into the anterior part of the HF, which could account for the residual hand2 expression (Fig. 1B, bracket). This might reflect an inadequate dose of SU5402 or, alternatively, the need for modulation of additional signals. Nevertheless, we show that exclusion of the haemangioblast programme from the HF is due, at least in part, to Fgf signalling.

To determine which Fgf ligands are responsible for our findings, and at the same time control for off-target effects of SU5402 (Mohammadi et al., 1997) and provide an independent genetic confirmation of the role of Fgf, we specifically knocked down expression of the three Fgf ligands that are expressed at the right time and place to be involved (Furthauer et al., 2004). Fgf8 is a prime candidate for controlling the equilibrium of the haemangioblast versus cardiac programme (Znosko et al., 2010). However, the extent of the phenotype observed in SU5402-treated embryos is clearly much stronger than that observed in the ace mutant. Making use of previously reported translation blocking antisense MOs (Fischer et al., 2003; Furthauer et al., 2001; Phillips et al., 2001), we separately knocked down the expression of fgf3, fgf8 and fgf24. Embryos injected with fgf24 MO displayed normal expression for the analysed ALPM genes (data not shown), whereas lack of pectoral fins at 72 hpf provided a positive control for the morpholino (data not shown) (Fischer et al., 2003). By contrast, when fgf3 was depleted, 7-somite embryos showed a reduction in cardiac marker expression, most clearly for nkx2.5, and an upregulation of blood and endothelial markers (see Fig. S1C,D in the supplementary material, arrows), albeit very mildly compared with embryos treated with SU5402 from 3 hpf (Fig. 1B,C and Fig. 2). When fgf8 was knocked down, a significant downregulation of cardiac marker expression was observed, coupled with an upregulation and expansion of haemangioblast gene expression (see Fig. S1C,D in the supplementary material, arrows and brackets). Thus, fgf8 seems to be playing a major role, with fgf3 making a more minor contribution and fgf24 apparently not involved. When fgf3 and fgf8 were knocked down at the same time, a strong reduction of cardiac gene expression, concomitant with a greater upregulation and expansion of blood and endothelial gene expression, was observed compared with either MO injected alone (see Fig. S1C,D in the supplementary material, note embryo percentages), and comparable with values achieved in 3 hpf SU5402-treated embryos (Fig. 2). Loss of rhombomere 5 provided a control for morpholino efficiency (Walsh et al., 2002) (see Fig. S1C,D in the supplementary material, see fgf3+8 MO). The addition of fgf24 MO to the fgf3-fgf8 double morphants did not alter the magnitude of the phenotype observed (data not shown). Thus, fgf3 and fgf8 together activate the cardiac programme and suppress the haemangioblast programme.

**Fgf influences haemangioblast and cardiac cell numbers during gastrulation**

The data above demonstrate that Fgf determines the relative numbers of haemangioblast and cardiac progenitors. To determine the timing of the Fgf requirement in this process, we inhibited signalling from various developmental time points and assayed for expression of one marker for each territory, namely etsrp (ets2 – Zebrafish Information Network) and nkx2.5, at the 7-somite stage, scoring the proportion of embryos displaying an increase or a decrease, respectively (Fig. 2). Reducing Fgf signalling from blastula and gastrula stages clearly expanded etsrp expression in the ALPM, including into the HF (Fig. 2). However, when blocked from late gastrula stages (75-90% epiboly and tailbud), the effect became weaker, and no significant differences from untreated embryos were detected when the treatment was performed from somitogenesis (2-3 somites) stages onwards.

A parallel decrease in cardiac (nkx2.5) expression was observed when Fgf signalling was inhibited from pre-gastrula and gastrula stages (Fig. 2). Approximately 90% of the analysed embryos showed a severe nkx2.5 downregulation when exposed to SU5402 at high (3 hpf), sphere, and 50% and 75-90% epiboly stages. A reduction in the effect on nkx2.5 expression was seen when Fgf was blocked later, albeit a little less dramatically than the effect seen on etsrp expression. To confirm the limited time window for affecting these populations, we blocked Fgf signalling from the 5-somite stage, when expression of both programmes had commenced, and no disruption of expression patterns was seen at the 10-somites stage (see Fig. S2A in the supplementary material). As a positive control, cmiely::GFP transgenic embryos treated with SU5402 at the 5-somite stage and collected at 48 hpf, showed a significant reduction in the size of the ventricle, whereas the atrial chamber was unaffected (see Fig. S2B in the supplementary material), in accordance with previously reported studies (Marques et al., 2008). We therefore conclude that, in the zebrafish embryo, Fgf signalling is required at gastrulation stages to determine the relative sizes of the cardiac and anterior haemangioblast fields, and that, once established, this decision cannot be overridden.

**Loss of heart progenitors and gain of haemangioblasts is not the result of apoptosis or proliferation**

The concomitant loss of heart cells and gain of haemangioblasts suggests a cell fate conversion, but an alternative explanation would be that one population is dying and the other is expanding. To test this, we monitored apoptosis and proliferation. As we have shown that Fgf acts during gastrulation, cell death was analysed at
Fig. 3. Fgf induces cardiac specification independently of, but concomitant with, haemangioblast repression. (A) Neither apoptosis nor proliferation is increased in SU5402-treated zebrafish embryos. Neither TUNEL nor phospho-histone-H3 (PHH3, green) staining increased in Fgf-depleted embryos during gastrulation or at the 5-somite stage. Nuclei are stained with DAPI (blue). (B) Fgf inhibition of haemangioblast gene expression precedes nkx2.5 expression. When Fgf was reduced, the haemangioblast programme was upregulated in the anterior lateral plate mesoderm (ALPM) before the onset of nkx2.5 expression. Embryos were treated from 3 hpf with SU5402 and RNA expression was assayed at the 3-somite stage. No differences were observed for nkx2.5 or pu.1, compared with DMSO-treated embryos, but scl and etsrp were upregulated in the ALPM (arrows). etsrp was also ectopically expressed in adjacent mesoderm (brackets). (C) scl and etsrp morpholino (MO) coinjection led to severe downregulation of anterior haemangioblast gene expression (pu.1 and scl) with associated rostral expansion of cardiac hand2 expression (arrowheads). nkx2.5 expression was unchanged at this stage but, when Fgf signals were also deficient in scl/etsrp double morphants (SU5402 + scl/etsrp MO), nkx2.5 expression was absent (arrows). Downregulation of hand2 expression in the cardiac region was also seen; however, rostral expression was still observed (arrowheads). (D) Fgf signalling is required concomitantly for the establishment of cardiac and haemangioblast fates. Fgf was inhibited for different periods of time and expression of etsrp and nkx2.5 assessed by in situ hybridisation at the 7-somite stage. Phenotypes were categorised as wild type (blue), type 1 (purple) or type 2 (yellow) (see inset key diagram), where type 1 represents downregulation of nkx2.5 with associated expansion of etsrp, and type 2 embryos show strong downregulation or absence of nkx2.5 and coupled expansion and upregulation of etsrp. Percentages of embryos showing increased etsrp or decreased nkx2.5 expression are shown. The experiment was repeated three times for 10 μM treatments and a representative experiment is shown, n>70 for each experimental point. Images show flat-mounted embryos, anterior to the top. Double-staining for myoD and krox20 (red) was used for staging and orientation. Scale bars: 100 μm.

Heart-haemangioblast antagonism

To determine whether the expansion of haemangioblast gene expression seen when Fgf is blocked is due to proliferation of the resident haemangioblast population, treated and untreated embryos were subjected to immunohistochemistry for phospho-histone-H3, a protein produced by dividing cells (Hendzel et al., 1997; Strahl and Allis, 2000) (Fig. 3A). No significant increase in proliferation was registered at 50% epiboly, 80% epiboly, tailbud or the 5-somite stage in embryos deficient for Fgf signalling, when compared with controls. Therefore, the increase in numbers of blood and endothelial cell progenitors cannot be explained by a higher proliferation rate.

Taken together with the unaffected numbers of cells expressing gata6 and nkx2.7 in the ALPM, these observations support a role for Fgf signalling in controlling the fates of cells in the ALPM, thereby determining the relative numbers of heart and anterior haemangioblast precursors in the developing embryo.

Fgf concomitantly inhibits haemangioblast and favours myocardial development

Previous work has shown that depletion of haemangioblast gene expression in the ALPM leads to an upregulation of cardiac gene expression, whereas over expression of haemangioblast regulators...
The anterior haemangioblast and cardiac programmes are cross-antagonistic

The observation that Fgf limits haemangioblast gene expression within the haemangioblast territory itself, and within the cardiac field regulates atrial and ventricular fates in a dose-dependent manner, indicates that it is working in a graded fashion. One mechanism by which graded morphogens are thought to establish all-or-nothing switches in cell fate and, thereby, sharp boundaries between territories is by cross-antagonism between the regulators induced at high and low concentrations (Ashe and Briscoe, 2006; Graf and Enver, 2009). Antagonism of the cardiac programme by the haemangioblast programme has been demonstrated by gain- and loss-of-function experiments (Gering et al., 2003; Schoenebeck et al., 2007); however, there is currently no evidence that the cardiac programme antagonises the haemangioblast programme. Indeed morpholino knockdown of nkh2.5, as well as having no effect on cardiac marker expression (see Fig. S2C in the supplementary material) as reported previously (Tu et al., 2009), did not lead to expression of haemangioblast markers in the heart field (see Fig. S2D in the supplementary material). Therefore, in the light of evidence for redundancy within the Nkh2 family (Fu et al., 1998; Tu et al., 2009), we took a gain-of-function approach, expressing nkh2.5 ectopically from injected RNA. Expression of the haemangioblast markers etsrp, scl and pu.1 at the 7-somite stage was dramatically downregulated, whereas hand2 expression was expanded into haemangioblast territory (Fig. 4A). Together with the continued expression of the ALPM marker gata6b, these data suggest that the cardiac programme might have been induced in cells fated to be anterior haemangioblasts. This change in gene expression profile was still evident at the 12-somite stage (data not shown). Thus, in addition to the inhibitory effect of haemangioblast regulators on the cardiac programme, cardiac regulators antagonise the haemangioblast programme, indicating that the programmes of these two populations are cross-antagonistic, a characteristic of lineage switches in eukaryotes (Graf and Enver, 2009).

Reprogramming of cardiac and haemangioblast cells by Fgf but not Nkh2.5 is maintained in differentiated tissues

To determine whether the reduction of cardiac and expansion of anterior haemangioblast fields on Fgf ablation, observed at the onset of their specification, reflects a genuine reprogramming of cardiac to blood/endothelial fates, we investigated whether more blood and endothelium accompanied loss of myocardium later in development. Endocardial and head vessel progenitors express the endothelial genes kdr and VE-cadherin (cdh5 – Zebrafish Information Network) and the endocardial progenitors start migrating from the ALPM at the 14-somite stage and fuse at the midline between the 15- and 18-somite stages (Bussmann et al., 2007) (Fig. 4B, arrow). When embryos were exposed to SU5402 from 3 hpf, an increase in the number of endothelial precursors expressing kdr or cdh5 was observed at the 16-somite stage (Fig. 4B). Based on location, this increase appears to be endocardium and associated endothelium, although in the absence of a zebrafish endocardium-specific marker this cannot be confirmed. A similar result was seen at the 20-somite stage (data not shown). To test the effects beyond this, kdr::GFP transgenic embryos were treated with SU5402 from 3 hpf and collected at 36 hpf, when an increase in the number of differentiated endothelial cells was clearly apparent (Fig. 4C, green), whereas cardiac cells positively stained for MF20 antibody were clearly reduced (Fig. 4C, red, arrows), as previously described (Marques et al., 2008). A similar increase in the number of myeloid cells, as revealed by pu.1 expression at the 16- and 20-somite stages and l-plastin (lcp1 – Zebrafish Information Network) expression at 30 hpf, was also clearly evident (Fig. 4B,D; data not shown). Furthermore, when embryos were only exposed to SU5402 during gastrulation, the period when Fgf signals are crucial for the proposed effects on heart and haemangioblast programmes, an excess of blood and endothelial gene expression was still observed at the 16- and 20-somite stages (data not shown).

To confirm, for endothelium in particular, that the apparent increase/decrease in ALPM cell fates (Fig. 4B) is not just due to cell migration defects, we performed quantitative PCR on tissue dissected from the heart region of control and Fgf-deficient embryos at 36 hpf. A clear reduction of cmhc2 (myl7 – Zebrafish...
Information Network) expression, together with an increase in pu.1 and kdrl expression, was observed ($P < 0.0005$ for all genes) (see Fig. S3A in the supplementary material). We therefore conclude that the increase in haemangioblast and decrease in cardiac precursor numbers seen in earlier embryos indicates genuine reprogramming reflected in the appearance of increased numbers of mature myeloid and endothelial/endocardial cells and decreased numbers of cardiomyocytes in later embryos.

Nkx2.5 expression is dependent on Fgf signalling (Fig. 3C) and Nkx2.5 overexpression, like Fgf inhibition, alters the ratio of haemangioblast to cardiac precursors (Fig. 4A). To determine whether nkx2.5 could be the mediator of the stable Fgf signalling effects we see, we monitored cardiac and blood/endothelial differentiation in nkx2.5 over-expressing embryos. The loss of myeloid programming, as revealed by the expression of the myeloid marker pu.1, at the 16- and 20-somite stages (data not shown), was maintained through to 26 hpf as revealed by the loss of l-plastin expression (Fig. 4E). However, by contrast, the earlier expansion of cardiac and loss of endothelial gene expression was not maintained. An increase of cmlc2 was not apparent at the 20-somite stage (data not shown) or 26 hpf and, although a decrease of kdrl and cdh5 at 20S was detectable, it was minimal (data not shown), and by 26 hpf it was not evident (Fig. 4E). Taken together, our data indicate that nkx2.5 cannot alone mediate all the effects of Fgf.

Lycat and Nkx2.5 mediate Fgf signalling to establish cardiac and haemangioblast identity

Because Fgf suppresses the expression of haemangioblast regulators at the same time as inducing the cardiac programme, we were interested to determine whether simultaneous manipulation of the two programmes by other means could achieve stable reprogramming. Loss of etsrp and scl together with gain of nkx2.5 was an attractive combination but none of the three regulators has been reported to be expressed during gastrulation when Fgf has its effects (Lee et al., 1996; Patterson et al., 2005; Sumanas and Lin, 2006). We therefore tested by qPCR the expression of these three genes during gastrulation and found that although nkx2.5 could be detected and was indeed suppressed by SU5402, etsrp and scl were at the limits of detection (Fig. 5A; data not shown). By contrast, the candidate cloche gene, lycat, lies upstream of both etsrp and scl in the haemangioblast genetic cascade (Xiong et al., 2008) and
DISCUSSION
Fgf drives cardiac fate at the expense of blood/endothelial fate in developing embryos

Our findings identify a novel role for Fgf in regulating cardiac and blood/endothelial fates in the anterior lateral plate mesoderm (ALPM) of zebrafish embryos. The absence of significant effects on proliferation or cell death, coupled with reciprocal consequences for the two lineages, makes a fate change the most likely interpretation of the phenotype of Fgf-inhibited embryos. We show that this fate decision is taken during gastrulation and that, once taken, it cannot be reversible by later manipulation of Fgf. Furthermore, this fate change is stable through later development ensuring that altered numbers of precursors for the two tissues are reflected in altered numbers of differentiated cells. Specific morpholino depletion shows that two of the Fgf ligands expressed at the right time and place in the embryo, namely fgf3 and fgf8, are responsible. fgfr1 and fgfr4 are widely expressed throughout the blastoderm of the zebrafish gastrula (Ota et al., 2010; Scholpp et al., 2004; Thisse et al., 1995), making it likely that the signalling is through these receptors.

It is known that Fgf signalling is active during gastrulation when our data indicate that it is controlling cell fate (Fischer et al., 2003; Furthauer et al., 2004; Phillips et al., 2001). A previous study had identified a role during gastrulation for Fgf signalling in controlling overall heart size (Marques et al., 2008), but a reciprocal and concomitant effect on the anterior haemangioblast programme had not been realised. A greater requirement for Fgf in ventricular development compared with atrial development was also identified in these earlier studies (Marques et al., 2008; Reifers et al., 2000) and we have confirmed this (see Fig. S2B in the supplementary material). Furthermore, the upregulation of blood and endothelial marker expression in the haemangioblast field itself indicates that Fgf is restricting blood and endothelial gene expression here too (Fig. 1C). Taken together, a picture emerges of a graded response and set up tissue boundaries is by cross-antagonism which a graded morphogen such as Fgf can elicit all-or-nothing responses and suppress the former and favouring the latter. One mechanism by which a graded morphogen such as Fgf can elicit all-or-nothing responses and set up tissue boundaries is by cross-antagonism between the regulators driving the two alternative outcomes (Ashe and Briscoe, 2006; Graf and Enver, 2009). Consistent with such a
mechanism, gain- and loss-of-function experiments have shown that the anterior haemangioblast programme is antagonistic towards the cardiac programme (Gering et al., 2003; Schoenebeck et al., 2007) (this study). However, loss-of-function experiments with the cardiac regulator \textit{nkx2.5} failed to reveal a role in suppressing haemangioblast gene expression (see Fig. S2D in the supplementary material). In view of reported redundancy within this gene family (Fu et al., 1998; Tu et al., 2009), we decided to take a gain-of-function approach and found that \textit{nkx2.5} does indeed have the ability to suppress the haemangioblast programme (Fig. 4A). We therefore conclude that the cardiac and haemangioblast programmes are indeed cross-antagonistic which might help to fix cell fates either side of the boundary between these two fields (Fig. 6B).

Although \textit{nkx2.5} over-expression can alter the balance between the blood/endothelium and heart progenitors early in development, an increase in differentiated cardiomyocytes was not observed in our experiments. This is in contrast to some extent with previously published data, which showed that over-expression of \textit{nkx2.5} led to a larger heart containing more cells (Chen and Fishman, 1996; Tu et al., 2009). However, in both the published studies, an enlarged heart was seen only at low frequencies and with only a modest increase in cell numbers (Chen and Fishman, 1996). Furthermore, Tu and colleagues also observed smaller hearts in a significant subset of embryos (Tu et al., 2009). The differences might reflect differing doses and/or stabilities of the mRNAs injected: in our experiments, \textit{nkx2.5} was fused to GFP so that its longevity could be tested, and we found that it could no longer be detected by the 15-somite stage, the approximate time at which recovery of the endothelial and cardiac programmes was observed (data not shown). This correlation might indicate that continued \textit{nkx2.5} expression is required to maintain cardiac and suppress endothelial fates.

The loss of reprogramming in our \textit{nkx2.5} over-expression experiments contrasts with the stable nature of the fate changes seen when Fgf was blocked, which suggests that \textit{nkx2.5} activity might not be solely responsible for the Fgf-mediated control over cardiac versus haemangioblast outcomes. Because a short burst of Fgf depletion during gastrulation still led to a stable change in the ratio of differentiated cell types (data not shown), something other than prolonging the \textit{nkx2} expression would appear to be necessary, although the function of this ‘something else’ could, in principle,
include maintenance of nkx2 expression. Here, we identify lycat as the possible additional regulator to nkx2.5; expression of lycat is affected by Fgf signalling during gastrulation and loss of lycat, and thereby the loss of its targets etsrp and scl, appears to be crucial to stable reprogramming of cardiomyocytes. Over-expression of nkx2.5 in a haemangioblast-deficient background allowed the early change in fate to be sustained and expression of cmklc2 and both atrial and ventricular gene expression to be stably increased. Thus, Fgf signalling simultaneously and independently induces the expression of nkx2.5 and represses the expression of lycat and, thereby, the key haemangioblast regulators etsrp and scl to specify the size of the heart field (Fig. 6B).

**Fgf signalling and expansion of the heart field during evolution**

Our studies show for the first time that Fgf restricts the anterior haemangioblast field while concomitantly inducing the heart field, ensuring the formation of appropriate numbers of both cell types. As inhibition of Fgf signalling can induce blood and endothelial specification in the cardiac mesoderm, we hypothesise that ectopic expression of Fgf signalling would have the reverse effect. By implanting beads soaked in FgR8 into post-gastrulation embryos, ectopic induction of cardiac gene expression has been demonstrated (Alsan and Schultheiss, 2002; Reifers et al., 2000). However, the Fgf role described in our studies occurs during pre-gastrula and gastrula stages, and attempts at gain-of-function at such early times, by injection of bFgf RNA into 1- to 2-cell stage embryos, resulted in severely dorsi-dorsalised embryos (data not shown) as shown previously (Furthauer et al., 1997). Similarly, Marques et al. were only able to study the consequences of constitutively active Fgf receptor expression after the 8–somite stage because of severe patterning defects earlier (Marques et al., 2008). By contrast, Molina et al. have recently reported an expansion of cardiac gene expression following Fgf agonist treatment of embryos, including during gastrulation (Molina et al., 2009). In addition to lateral expansions of gata4 and nkx2.5 expression, they were able to show an anterior expansion of hand2 expression at the expense of scl expression. We have been able to repeat this result only at very low frequency (data not shown), reflecting the difficulties in upregulating Fgf activity during gastrulation. Nevertheless, the ability to achieve such fate change anteriorly in the haemangioblast territory is entirely consistent with the predictions of our loss-of-function data. Likewise, in Drosophila, transgenic expression during gastrulation of an activated Fgf receptor (heartless) increased the size of the heart field (Michelson et al., 1998), and in Ciona intestinalis, when gastrula cells were treated with Fgf outside the embryo, cardiac differentiation was increased (Davidson et al., 2006). We therefore conclude that anterior enhancement of Fgf signalling during evolution could be expected to recruit presumptive anterior haemangioblasts into the heart field.

The acquisition of extra chambers in the heart by annelids during evolution is accompanied by loss of the anterior haemangioblast population (Dzierzak and Speck, 2008). Our observations here suggest a mechanism by which this fate change could have been achieved, namely by an increase in Fgf signalling (Fig. 6C). The newly recruited population of cardiac precursors led to the development of a larger heart with more chambers. In this view, amphibians, whose hearts have three chambers but who retain a residual anterior haemangioblast population (Walmsley et al., 2002), might represent an intermediate stage of the recruitment (Fig. 6C). Here, we have not monitored lower jaw muscle fates but recent observations in Ciona suggest that this lineage, which shares programming with cardiac muscle, might also have been expanded by recruitment of the anterior haemangioblast population (Stolfi et al., 2010). An interesting implication of this proposal with respect to the adult heart is that the much sought after property of multipotentiality in stem/progenitor cells could be more likely in candidates that have been more recently recruited from cells with endothelial potential.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**References**


