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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 isletT 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 gata*6* 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

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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 *nkx2.5* 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 *nkx2.5* are expressed during gastrulation in an Fgf-dependent manner. Furthermore, Fgf-independent repression of haemangioblast regulators together with overexpression of *nkx2.5* 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 represents a mechanism by which the anterior haemangioblast population could have been recruited into the heart field (HF) during evolution.

MATERIALS AND METHODS

Zebrafish strains

Wild-type (WT) and transgenic *kdrl*::GFP and *cmlc*$_2$::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), *fgf8* (Furthauer et al., 2001), *fgf24* (Fischer et al., 2003), *scl* (Patterson et al., 2005), *etsrp* (Sumanas and Lin, 2006) and *nkx2.5* (Sultana et al., 2008) MOs were injected at published levels and embryos were fixed at described stages.

Nkx2.5-GFP plasmid generation and RNA injection

Nkx2.5 PCR fragments were generated via Superscrip III One-Step RT-PCR System (Invitrogen), using total RNA extracted from 5-somite embryos, with the following primers (5′-3′): *nkx2.5*-gateway-Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCAATGTTCT-TAGCC; *nkx2.5*-gateway-Reverse: GGGGACCACTTTGTACAAG-AAAGCTGGGTCCCAAGCTCTGATGCCATG. Gateway cloning technology (Invitrogen) generated an *nkx2.5* entry vector in pDONR221 backbone, which was recombined with pCSGFP2 (Gering et al., 1998) to create an *nkx2.5*-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 by an Alexa 555 goat anti-mouse 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. Gapdh 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 supplementary 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).

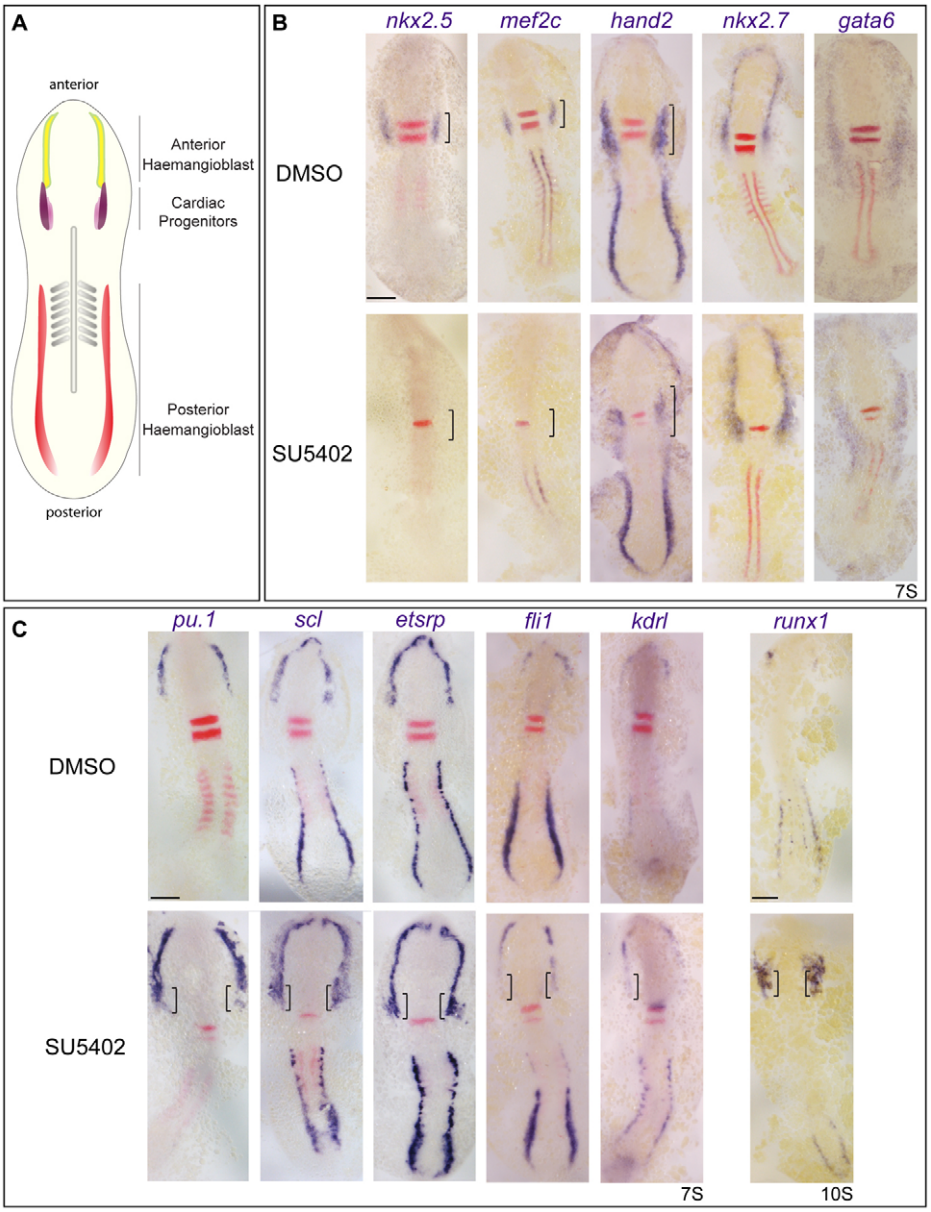

Fig. 1. Fgf signalling inhibits blood and endothelial gene expression in the heart field. (**A**) Schematic highlighting the anterior blood/endothelial precursor or haemangioblast population (yellow) rostral to cardiac precursors (purple) in 7-somite zebrafish embryos. Another haemangioblast population is found in the posterior lateral plate mesoderm (red). (**B**) Expression of the cardiac markers (brackets) *nkx2.5*, *mef2c* and *hand2* is severely reduced in embryos treated with the Fgf inhibitor SU5402 compared with control DMSO-treated siblings. A few *hand2*$^{+}$ cells remain in the cardiac mesoderm of inhibitor-treated embryos. Anterior mesoderm specification is unaffected as revealed by normal expression of the anterior lateral plate mesoderm (ALPM) markers *nkx2.7* and *gata6*. (**C**) Blood and endothelial gene expression is upregulated and ectopic (brackets) in the ALPM of SU5402-treated embryos. Images show flat-mounted embryos, anterior to the top. Somites were stained with *myoD* or *uncx4* (red) for staging. Rhombomeres 3 and 5 were stained with *krox20* (red stripes) as a positional marker. Reduction in rhombomere 5 reveals that Fgf signalling is reduced, together with short body axis and posterior mesoderm defects. Scale bars: 100 μm.

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). *mef2c* is present in the pre-myocardial mesodermal cells (Ticho et al., 1996) and *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 *hand2*$^{+}$*nkx2.5*$^{-}$ cells remaining in the Fgf-deficient embryos, possibly reflecting the lower dependence of atrial cell fate on Fgf (Marques et al., 2008; Reifers et al., 2000; Schoenebeck et al., 2007). This reduced cardiac gene expression did not result from loss of cells, because expression of both *gata6* and *nkx2.7*, which are normally expressed in both the cardiac and haemangioblast fields, was unaltered (Peterkin et al., 2009; Reifers et al., 2000) (Fig. 1B). The reduction of *nkx2.5* expression and lack of significant effect on *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 *nkx2.5* expression reported previously (Reifers et al., 2000), and that the concomitant reductions we observed for *mef2c* and *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 *pu.1* (*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

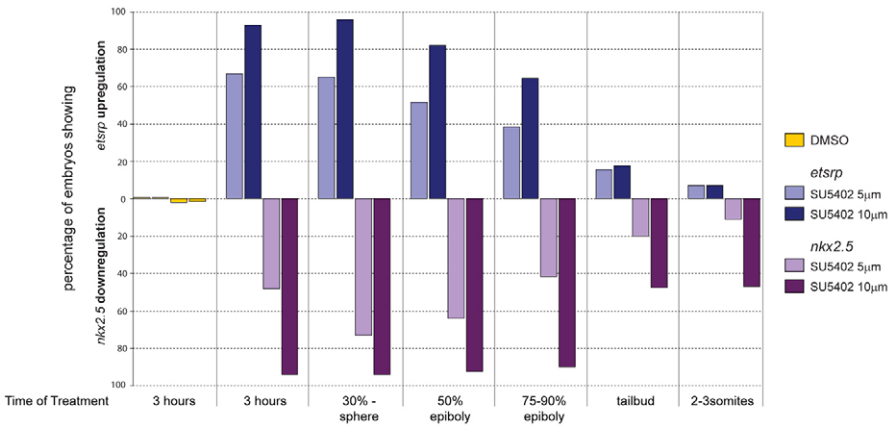

Fig. 2. Fgf determines cardiac versus blood/endothelial cell numbers during gastrulation. Fgf was inhibited from various time points and expression of *etsrp* (blue bars) and *nkx2.5* (purple bars) in the anterior lateral plate mesoderm (ALPM) was assessed by in situ hybridisation in 7-somite stage zebrafish embryos. Note the greater effect at pre-gastrula and gastrula stages. The experiment was repeated three times each for 5 μM and 10 μM treatments. Affected embryo percentages from one representative experiment are shown, *n*>70 for each experimental point.

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 (Walshe 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* (*etv2* – 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, *cmlc*$_2$::*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

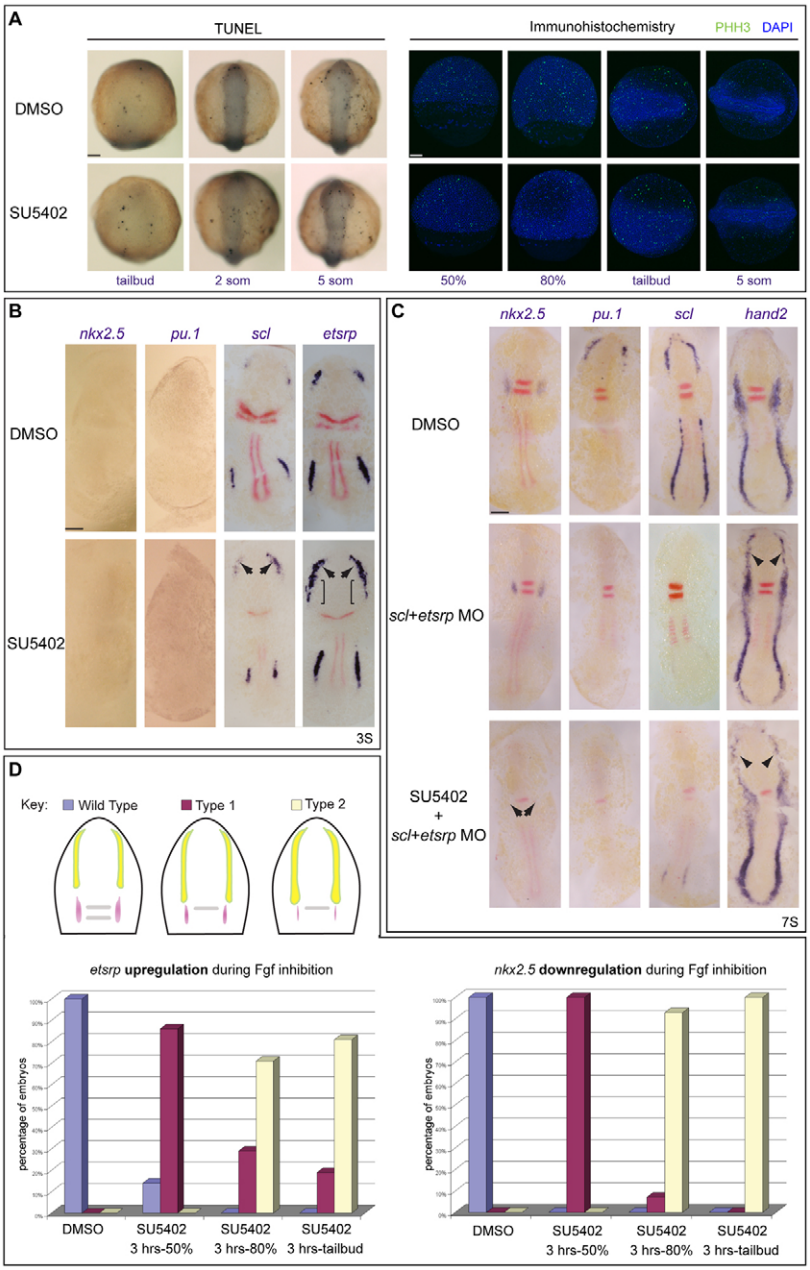

Fig. 3. Fgf induces cardiac specification independently of, but concomitant with, haemangioblast repression. (**A**) Neither apoptosis nor proliferation is increased in SU5402-treated zebrafish embryos. Neither TUNEL nor phospho-histone-H3 (PHH3, green) staining increased in Fgf-depleted embryos during gastrulation or at the 5-somite (5 som) 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) co-injection 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.

this time by TUNEL staining. As reported previously (Yamashita, 2003), no apoptosis was evident during gastrulation of wild-type embryos and, importantly, it was not induced in embryos exposed to SU5402 (data not shown). Because we have shown that the effects of Fgf loss are apparent during early somitogenesis, we also monitored apoptosis during early somite stages and found that a few apoptotic cells were visible but no significant increase was detected in Fgf-depleted embryos (Fig. 3A). These data are in line with several studies that show no increase in apoptosis on depletion of Fgf signalling at various stages of zebrafish development (Maroon et al., 2002; Marques et al., 2008). Thus, loss of cardiac cells does not appear to result from increased apoptosis in the ALPM of SU5402-treated embryos.

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

suppresses it (Gering et al., 2003; Schoenebeck et al., 2007). As the known haemangioblast regulators were thought to be expressed before the known cardiac regulators, we wondered whether the loss of cardiac precursors on depletion of Fgf was a consequence of gain of haemangioblast regulator expression, or whether Fgf also positively regulates the heart programme. Support for such a notion came from the observation that *etsrp* was already expressed in the adjacent presumptive cardiac mesoderm in Fgf-inhibited embryos at the 3-somite stage (Fig. 3B, brackets). In addition, both *scl* and *etsrp* were already upregulated within the anterior haemangioblast territory at this time (Fig. 3B, arrowed). Thus, the absence of cardiac tissue in SU5402-treated embryos could indeed be due to an expansion of the repressive anterior haemangioblast programme.

To determine whether this is how loss of Fgf suppresses the cardiac programme, we utilised previously published *scl* and *etsrp* morpholinos (MO) (Patterson et al., 2005; Sumanas and Lin, 2006) to independently inhibit the induction of the haemangioblast programme in SU5402-treated embryos. As previously described, *pu.1* and *scl* were downregulated in *scl+etsrp* morphants, and *hand2* was upregulated into the rostral ALPM (arrowhead) (Patterson et al., 2005; Schoenebeck et al., 2007; Sumanas and Lin, 2006) validating the morpholino doses used (Fig. 3C). However, unlike *hand2* expression, *nkx2.5* expression did not expand, suggesting that these haemangioblast regulators are not regulators of *nkx2.5* expression. Importantly, though, *scl+etsrp* morphant embryos treated with SU5402 (SU5402 + *scl+etsrp* MO) had little or no expression of *nkx2.5* (Fig. 3C), indicating that its expression is still dependent on Fgf signalling even in the absence of *scl* and *etsrp*. Thus, Fgf is indeed required for cardiac specification independently of its role in haemangioblast suppression.

To determine whether the effects on the two programmes occur simultaneously or consecutively, we treated embryos with short bursts of SU5402 for differing lengths of time. Application was at 3 hpf and the inhibitor was then washed out at 50% or 80% epiboly or at tailbud stages, and *etsrp* and *nkx2.5* expression was analysed at the 7-somite stage. The time required for total recovery from SU5402 treatment has been shown to be ~2 hours (Crump et al., 2004; Maroon et al., 2002; Marques et al., 2008; Nechiporuk et al., 2005). The phenotypes of the embryos were scored as wild type; type 1: downregulation of *nkx2.5* and expansion of *etsrp* posteriorly; or type 2: severe downregulation/absence of *nkx2.5* and posterior expansion together with anterior upregulation of *etsrp* (see Fig. 3D, key). The severity of phenotypes, for both haemangioblast and cardiac markers, increased in the same way with the length of treatment (Fig. 3D). Thus, the effects of Fgf on the haemangioblast and cardiac programmes occur at the same time, consistent with Fgf operating a cell-fate switch.

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 *nkx2.5*, 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 Nkx2 family (Fu et al., 1998; Tu et al., 2009), we took a gain-of-function approach, expressing *nkx2.5* 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 *gata6*, 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 Nkx2.5 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 *kdrl* 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 *kdrl* 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, *kdrl*::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 *cmlc2* (*myl7* – Zebrafish

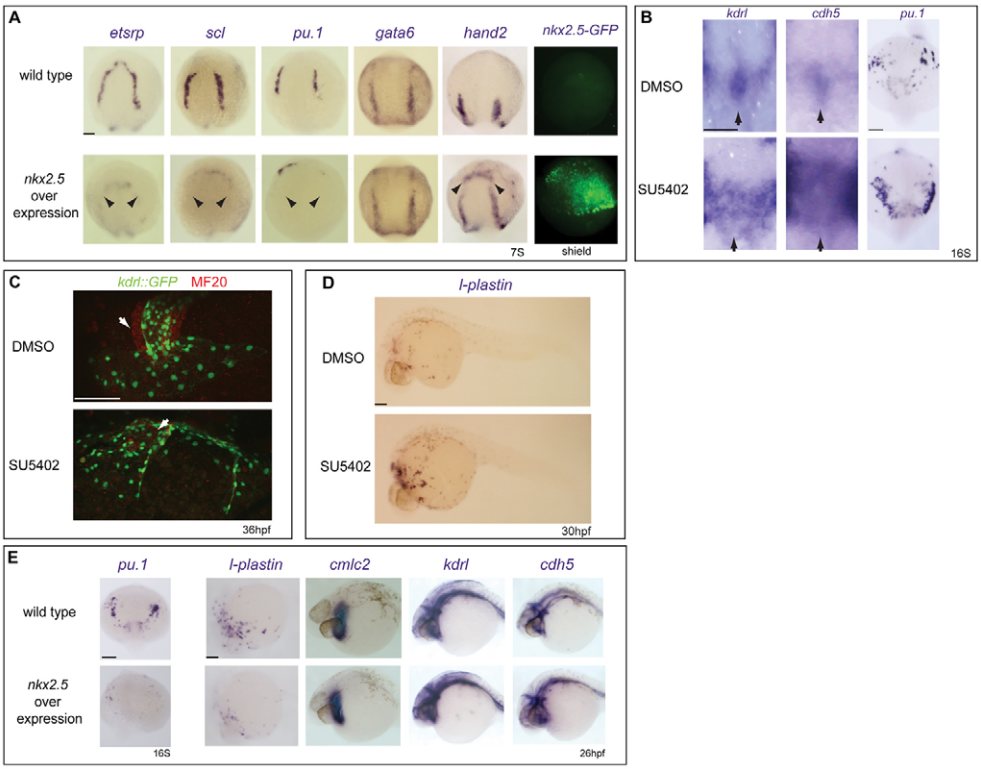

Fig. 4. The cardiac and blood/endothelial programmes are cross-antagonistic. (**A**) *Nkx2.5* RNA over-expression (50 pg) from the 1- to 2-cell stage reduced *etsrp*, *scl* and *pu.1* and expanded *hand2* expression in the anterior lateral plate mesoderm (ALPM; arrowheads) at the 7-somite stage. The anterior mesodermal marker *gata6* was unaffected in the same cells. *Nkx2.*5-GFP fusion expression was visualised at the shield stage to confirm injection and expression. Dorsal views of 7-somite whole zebrafish embryos are shown, anterior to the top; for fluorescence, lateral views of shield stage whole embryos are shown, dorsal to the right. (**B**) An increase in endothelium and myelopoiesis was evident throughout development upon depletion of Fgf. Embryos treated with SU5402 at 3 hpf were assessed for endocardium by expression of *kdrl* and *cdh5* at the 16-somite stage, whereas *pu.1* was assessed for myeloid development. Increased endocardial (arrows) and myeloid gene expression was observed. (**C**) Similar treatment increased *kdrl*::GFP^{+} endothelial cell numbers, with concomitant loss of myocardium at 36 hpf (white arrow; stained with MF20 antibody). (**D**) Increased differentiated myeloid cells (*l-plastin*$^{+}$) seen at 30 hpf. (**E**) *Nkx2.*5-induced downregulation of the haemangioblast programme is not sustained throughout development. Myeloid gene expression (*pu.1*) was downregulated at the 16-somite stage upon *nkx2.5* over-expression, and at 26 hpf *l-plastin* remained depleted. However, expression of *cmlc2*, *kdrl* and *cdh5* appeared similar to wild type at 26 hpf. Dorsal views are shown, anterior to the top, except in panel D and 26 hpf embryos in panel E in which lateral views are shown, anterior to the left. Scale bars: 100 μm.

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

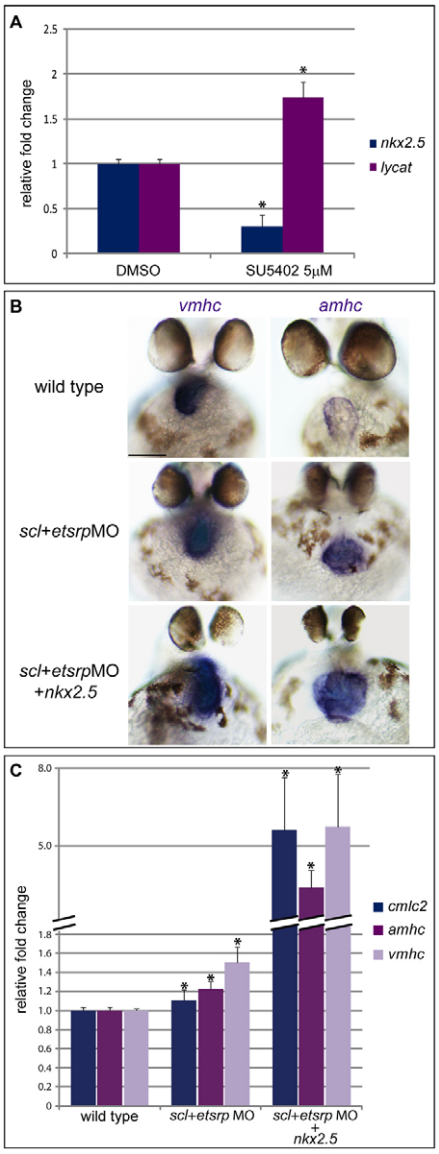

Fig. 5. Fgf is working via *nkx2.5* and *lycat* during gastrulation to establish cardiac and haemagioblast fates. (**A**) Quantitative PCR on cDNA obtained from RNA extracted from 50% epiboly zebrafish embryos treated with DMSO and SU5402, assayed for *lycat* and *nkx2.5* expression normalised to *gapdh*. Shown are fold changes in expression, relative to the DMSO control, from three biological replicates. Error bars indicate s.e.m., asterisks indicate statistically significant differences (P<0.001) compared with DMSO controls. (**B**,**C**) The *scl* and *etsrp* morpholino combination increased *amhc* and *vmhc* expression, as seen by in situ hybridisation (B) and qPCR (C; P<0.02) at 48 hpf. However, expression was substantially more upregulated in *scl+etsrp*MO and *nkx2.5* RNA embryos (P<0.05). A similar trend was observed for *cmlc2* (P<0.05). Three biological replicates were analysed. Error bars indicate s.e.m., asterisks indicate statistically significant differences compared with wild type and *scl+etsrp*MO siblings. Scale bars: 100 μm.

qPCR showed that it was both expressed and enhanced by SU5402 (Fig. 5A), thus supporting the notion that *lycat* together with *nkx2.5* could be candidate responders to Fgf signalling (Fig. 5A).

We therefore wished to determine whether overexpression of *nkx2.5* in haemangioblast-deficient embryos could lead to a stable increase in cardiomyocyte numbers. Over-expressing *nkx2.5* mRNA in *cloche* mutant embryos was an option but only 25% of the offspring carry the mutation. We therefore utilised the *scl* and *etsrp* morpholinos in combination, which phenocopies the *cloche* mutant (Patterson et al., 2005; Schoenebeck et al., 2007; Sumanas et al., 2008). *scl* and *etsrp* morphants showed some increase in *amhc* and *vmhc* compared with the controls (Fig. 5B, 48 hpf) supported by qPCR (P<0.02 for both genes) (Fig. 5C). However, expression of *amhc* (*myh6* – Zebrafish Information Network) and *vmhc* were substantially upregulated in the *scl+etsrp* morphants in which *nkx2.5* was over-expressed (P<0.05 for both genes) (Fig. 5B,C). A similar trend was observed for *cmlc2* (P<0.05) (Fig. 5C and see Fig. S3B in the supplementary material). We therefore conclude that Fgf is working during gastrulation through repression of the haemangioblast regulator *lycat* and concomitant induction of *nkx2.5*.

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 reversed 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 dependence on Fgf signalling whereby ventricular fate is most dependent on Fgf, with atrial differentiation requiring less, and anterior blood/endothelium less still (Keegan et al., 2004; Warga et al., 2009) (Fig. 6A).

Differential Fgf responses and cross-antagonism

Our experiments place Fgf signalling at the top of the anterior haemangioblast and cardiac programmes, playing a dual role in suppressing 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

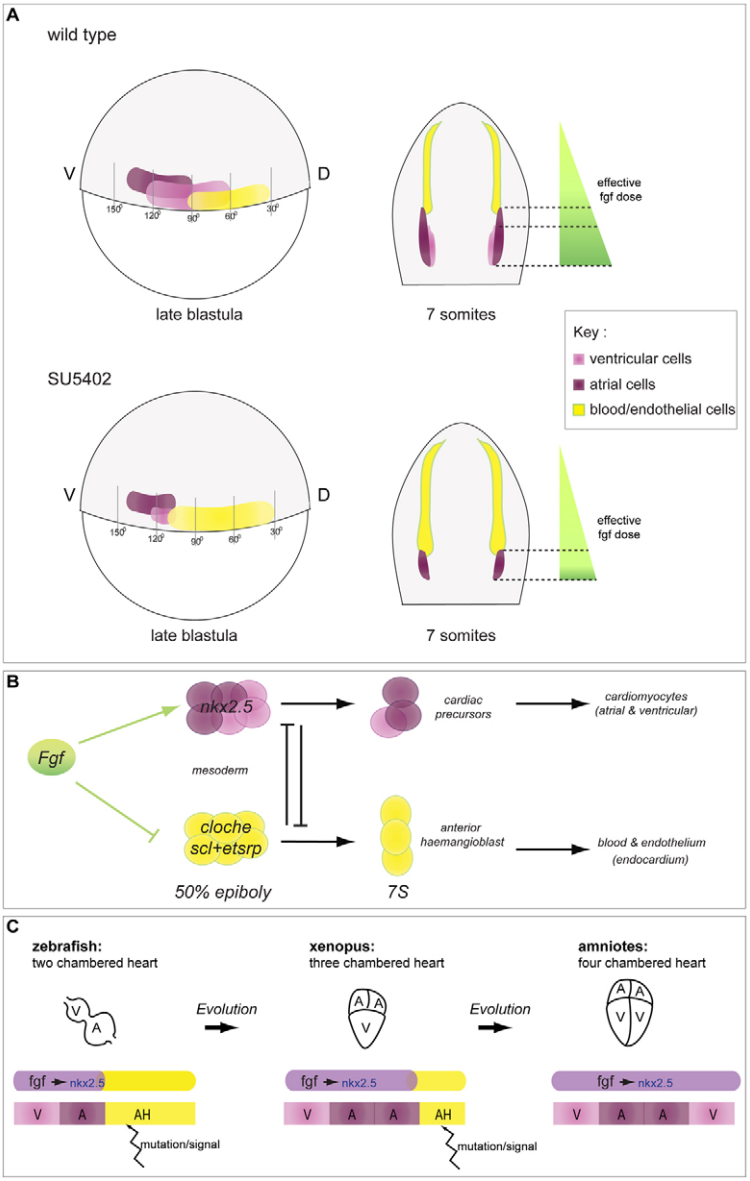

Fig. 6. The role of Fgf in zebrafish heart formation and, potentially, in evolution. (**A**) Schematic of the consequences of reduced Fgf signalling on the establishment of the cardiac-blood/endothelial boundary and atrial/ventricular fates during gastrulation. D, dorsal; V, ventral. (**B**) Mechanism of Fgf action on the cardiac and haemangioblast programmes. 7S, 7 somites. (**C**) Model for heart evolution. Increasing the domain of Fgf signalling sequentially recruits the anterior haemangioblast (AH) into the heart field, resulting in a larger heart with increased number of chambers. A, atrium; V, ventricle.

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 *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 *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 *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 *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, *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 *nkx2.5* expression is required to maintain cardiac and suppress endothelial fates.

The loss of reprogramming in our *nkx2.5* over-expression experiments contrasts with the stable nature of the fate changes seen when Fgf was blocked, which suggests that *nkx2* 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 *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 *cmlc2* 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 Fgf8 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 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 amniotes 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, amphibia, 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 multi-potentiality 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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