Genomes & Developmental Control

Redundancy and evolution of GATA factor requirements in development of the myocardium

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Abstract

The transcription factors, GATA4, 5 and 6, recognize the same DNA sequence and are all expressed in the developing myocardium. However, knockout studies in the mouse have indicated that none of them are absolutely required for the specification of the myocardium. Here we present evidence for redundancy in this family for the first time. Using morpholinos in both *Xenopus* and zebrafish embryos, we show that GATA4 knockdown, for example, only affects cardiac marker expression in the absence of either GATA5 or GATA6. A similar situation pertains for GATA5 in *Xenopus* whereas, in zebrafish, *GATA5* (*faust*) plays a major role in driving the myocardial programme. This requirement for GATA5 in zebrafish is for induction of the myocardium, in contrast to the GATA6 requirement in both species, which is for differentiation. This early role for GATA5 in zebrafish correlates with its earlier expression and with an earlier requirement for BMP signalling, suggesting that a mutual maintenance loop for GATA, BMP and Nkx expression is the evolutionarily conserved entity.
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Introduction

The GATA factors are zinc finger transcriptional activators that bind to the consensus DNA sequence (A/T)GATA(A/G). They have been identified throughout eukaryotes and been shown to play critical roles in both haematopoiesis and cardiogenesis in vertebrates and *Drosophila* (Fossett and Schulz, 2001; Nemer and Nemer, 2001). Of the six evolutionarily conserved GATA genes in vertebrates, *GATA4*, *5* and *6* are expressed in the heart as it develops.

Loss and gain of function studies in P19 embryonal carcinoma cells indicated a requirement for GATA4 in the differentiation of cardiac restricted cells to beating cardiomyocytes (Grepin et al., 1997, 1995). In addition, overexpression of *GATA4* in *Xenopus* embryos and explants resulted in expression of cardiac differentiation markers and in some cases spontaneously beating tissue (Jiang and Evans, 1996; Latinkic et al.,

2003). However, in the *GATA4* null mouse, normal amounts of myocardial tissue appeared to be formed (Holtzinger and Evans, 2005; Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1996). Thus, even though cardia bifida and defects in looping morphogenesis were observed in the null mouse embryos, specification of the myocardium appeared to take place normally. A suggested explanation for this was the elevated expression of *GATA6* (Holtzinger and Evans, 2005; Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1996; Pu et al., 2004). Consistent with this proposed redundancy of function within the family, *GATA5* and *6* are also active in the P19 cell line and *Xenopus* explant assays described above. Thus, it appears that each of these three GATA family members possesses the capability of inducing cardiac differentiation in gain-of-function assays, however demonstration that they exhibit such redundancy *in vivo* awaits combinatorial loss-of-function assays.

The *GATA5* null mouse shows no cardiac phenotype, however it may not be a true knockout due to the potential formation of a truncated protein containing the DNA binding domain (Nemer and Nemer, 2002). In the zebrafish, a critical

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role for *GATA5* in specification of the myocardium has been demonstrated by loss and gain of function assays (Reiter et al., 1999). The *faust^tm236* mutant shows a severe reduction in expression of cardiac markers and injection of *GATA5* RNA induces ectopic expression of the same markers. However, *GATA4* expression in the zebrafish *faust^tm23* mutant is significantly reduced and overexpression of *GATA5* results in ectopic expression of *GATA4*. Thus, these studies raise the possibility that the *GATA5* knockdown phenotype is due to the combined loss of *GATA4* and *5*.

The *GATA6* null mouse is an embryonic lethal due to an extra-embryonic defect and chimeras have indicated that *GATA6* is not required for specification of the myocardium (Kabrun et al., 1997; Koutsourakis et al., 1999; Kuo et al., 1997; Molkentin, 2000; Molkentin et al., 1997; Morrisey et al., 1997). However, we have presented evidence that *GATA6* is required for the maintenance and differentiation of cardiac progenitors in zebrafish and *Xenopus* embryos (Peterkin et al., 2003). The likely resolution of these apparently contradictory data is that the major consequence of lost *GATA6* function is non-cell autonomous and can therefore be rescued by surrounding wild type cells in mouse chimeras. The likely non-cell autonomous target for *GATA6* is BMP (Peterkin et al., 2003). However, this requirement for *GATA6* is for differentiation of cardiac progenitors and not for their initial specification.

In this study we use antisense morpholinos in *Xenopus* and zebrafish embryos to deplete combinations of GATA4, 5 and 6 for the first time. This has allowed us to provide the first experimental support for redundancy *in vivo*. In addition, we show that the strong dependence of zebrafish on GATA5 is not mirrored in *Xenopus*, where this GATA factor plays only a redundant role, like GATA4 in both species. The requirement for GATA5 in the zebrafish is for the induction of the myocardial programme, whereas in *Xenopus*, GATA activity is only required for differentiation. We propose that the primary function for GATA factors in development of the myocardium is in creating a sub-circuit of the regulatory network, involving another critical transcription factor, Nkx, and a crucial signalling pathway, BMP. This mutually supportive sub-network is evolutionarily stable, even though where the network is initiated appears to be more flexible.

Materials and methods

In situ hybridisation of whole-mounted and sectioned embryos

Xenopus and zebrafish were maintained and embryos were raised and staged using standard conditions (Nieuwkoop and Faber, 1967; Westerfield, 1993). In situ hybridisations on whole-mounted and sectioned embryos were carried out as previously described (Ciau-Uitz et al., 2000; Jowett, 2001; Walmsley et al., 1994). All RNA probes used were labelled with digoxigenin (DIG) except for *MyoD* and *Krox20* which were used in double in situ hybridisations and labelled with fluorescein. Detection of the antibody–alkaline phosphatase was done using BM purple (Roche) or Fast red (Sigma). After in situ hybridisation, embryos were re-fixed in 4% paraformaldehyde, zebrafish embryos were transferred into 75% glycerol to be photographed. Cryostat sections were performed after in situ hybridisation, embryos were fixed as above and washed in 30% sucrose. Embryos were transferred into embedding chambers in O.C.T Compound (Tissue-Tek) and 30 μm sections were cut on a Leica CM3050S.

Morpholino (MO) injection

The GATA4/5/6 antisense morpholinos were designed and manufactured by Genetools. Morpholino sequences: *Xenopus* GATA4 MO 5′ctggcaactcaatcca-caaaatcca3′ (data shown here), a second morpholino designed against the same pseudo-allele as described by Afouda et al. (2005) (data not shown, 5′ agctatactctgatacatcctgatc3′), and a third GATA4 MO designed to block both pseudo-alleles (a kind gift from Todd Evans) (data not shown) gave the same results. Zebrafish GATA4 MO 5′gccatcgttacaccttgatacatat3′ or a second splice morpholino as described by Holtzinger and Evans (2005). For *Xenopus* GATA5 MO 5′gctacaaacctcacagctcc3′ see Afouda et al. (2005). Zebrafish splice GATA5 MO 5′tgttaagatttttacctatactgga3′. For *Xenopus* and zebrafish GATA6 MOs see Peterkin et al. (2003). MOs were diluted in deionised water and injected as described (Peterkin et al., 2003). For zebrafish, 25 ng GATA4 MO, 25 ng GATA5 MO and/or 5 ng GATA6 MO were injected into single-cell embryos individually and in combination. For *Xenopus* embryos, a total of 20 ng of GATA4 MO or GATA5 MO and 10 ng of GATA6 MO were injected individually or in combination.

Results

GATA6 is the only essential GATA activity in Xenopus myocardium

We have reported previously that GATA6 is required for the maintenance and maturation of cardiomyocytes in *Xenopus* (Peterkin et al., 2003). The phenotypes consequential upon depletion of GATA4 or 5 in *Xenopus*, however, have not been previously reported. In the case of GATA5, the need to know is made greater because of its major contribution in zebrafish, and the inability to determine if this is a general requirement in vertebrates by comparison with the mouse knockout, because the reported mutation in the mouse appears not to be a null (Nemer et al., 1999). Therefore, before examining depletion of combinations of GATA factors, we examined the individual loss of GATA4 and 5 in comparison to the already known GATA6 phenotype.

The design and quality control of MOs against *Xenopus* GATA4, 5 and 6 have been reported previously (Peterkin et al., 2003; Afouda et al., 2005). For GATA4, as well as the MO reported previously, two other MOs, one against both pseudo-alleles (Todd Evans, personal communication), were tested and gave the same results. For GATA5 and 6, MOs were designed to target both pseudo-alleles of the *Xenopus laevis* genes. The optimal amount of each MO injected was determined by titration to ensure that the maximum dose without non-specific effects was used. The extent of knockdown by these ATG MOs was determined by co-injection of tagged reporter RNAs followed by Western blotting (Afouda et al., 2005; Peterkin et al., 2003). Very little residual protein was detected in each case.

When MOs against individual GATA factors were injected separately into the presumptive heart field, the dorsolateral marginal zone, of 4-cell *Xenopus* embryos, cardia bifida was induced in each case (Fig. 1A, visualised by staining the cells for expression of *Myosin Light Chain 2* (*MLC*)). Cardia bifida has been reported previously for the *GATA4* knockout mouse (Kuo et al., 1997; Molkentin et al., 1997), the *GATA5* mutant zebrafish, *faust* (Reiter et al., 1999), and GATA6 morphant *Xenopus* and zebrafish embryos (Peterkin et al., 2003), but this

Fig. 1. Cardia bifida is evident in GATA4, 5 and 6 morphants, but cardiac gene expression is only affected in GATA6 depleted *Xenopus* embryos. (A) Cardiac tissue stained for *MLC* fails to migrate to the midline in embryos injected singly with GATA4, 5 or 6 morpholinos. (B, C) Expression levels of *MLC*, *Nkx2.5*, *Tbx5* and *CA* remain unchanged in GATA4 and 5 morphants compared with control uninjected embryos at stage 28. (D) Expression levels of *MLC*, *Nkx2.5*, *Tbx5* and *CA* are substantially decreased in GATA6 morphants at stage 28.

is the first direct comparison in a single species showing that all three GATA factors are required for the timely migration of cardiac precursors to the midline for fusion of the heart tube. This is in contrast to requirements in the differentiation of the myocardium (see below), where only one member of the family is essential. It seems likely that the requirement for GATA4, 5 and 6 in midline migration of myocardial precursors is actually in the underlying endoderm, where they are all expressed and

which has been shown to be essential in mouse and zebrafish for heart tube fusion (Afouda et al., 2005; Alexander et al., 1999; Molkentin et al., 1997; Narita et al., 1997; Reiter et al., 1999; Weber et al., 2000).

To determine the effects of the GATA MOs on programming of the myocardial cells, as opposed to their morphological movements, the levels of expression of the transcription factors, *Nkx2.5* and *Tbx5*, and of the contractile machinery genes,

cardiac actin (*CA*), and *MLC*, were monitored by whole mount in situ hybridisation. In contrast to the GATA6 MO, which causes a profound reduction in the expression of these genes (Peterkin et al., 2003) (Fig. 1D), GATA4 and GATA5 MOs had minimal effects (Figs. 1B, C; for all three MOs and for each marker n was 30–50). Despite the cardia bifida at tailbud stages (stages 28–32) (Fig. 1A), the gross morphology of the hearts at later stages (stage 43) in GATA4 and GATA5 morphants looked similar to those in wild type embryos, i.e. the cardia bifida was only transient (data not shown). In contrast, as previously described (Peterkin et al., 2003), little or no cardiac tissue was observed in embryos depleted of GATA6 (data not shown). Thus, it would appear that, apart from the transient bifida, the loss of GATA4 or GATA5 has little effect on cardiogenesis in *Xenopus*. To ensure that the GATA4 and 5 morpholinos were properly functional, they were injected vegetally at the single-cell stage, and were shown to reduce the expression of *Sox17α* during gastrulation (data not shown) (Afouda et al., 2005). Furthermore, the gut of GATA5 morphants failed to coil properly, as previously reported (Afouda et al., 2005). In addition, for these and several of the combination experiments described below, all three GATA4 MOs gave the same results. We therefore conclude that for development of the myocardium in *Xenopus* embryos, GATA6 is the only essential GATA factor.

GATA factor redundancy in Xenopus myocardium

On the basis of slightly increased expression of *GATA6* in *GATA4* knockout mice, redundant roles for the GATA factors in the myocardium have been suggested (Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1996; Watt et al., 2004). In *Xenopus*, expression of neither *GATA5* nor *GATA6* was significantly increased in GATA4 MO injected embryos (data not shown). Similarly, in GATA5 and GATA6 MO injected embryos: in neither case was an increase in expression of the other two GATA factors observed (data not shown). However, redundancy does not necessarily depend on an increase in expression of the redundant family member: continued expression could suffice, and that is what we see in all three cases. Therefore to formally test redundancy within the GATA family, we injected combinations of MOs into the presumptive heart field of 4-cell *Xenopus* embryos, and monitored *MLC* and *Nkx2.5* expression by whole mount in situ hybridisation (Fig. 2). Embryos were classified as unaffected (wild type, +), mildly (−) or strongly (− −) down regulated, or displaying no expression at all (− − −) (Fig. 2A). Numbers of embryos in each category were scored and the results displayed in histograms (n=31–94) (Figs. 2B, C). The greater effects of the GATA6 MO are immediately apparent, with clear increases in the affected categories at the expense of the wild type category compared to both control embryos and also to GATA4 or GATA5 MO injected embryos.

When combinations of two MOs were injected, evidence for redundancy was revealed (Figs. 2B, C). Despite having little effect on their own, both GATA4 and GATA5 MOs made the phenotype of GATA6 MO injected embryos more severe when injected with it. Furthermore, the phenotype observed when GATA4 and 5 MOs were injected in combination was significantly worse than either alone, suggesting that the minimal phenotype for the single injections relied on the continued presence of the other GATA factor. When all three MOs were injected together, the phenotype was the most extreme of all with the vast majority of embryos having no expression of *MLC* at all. We therefore conclude that, while GATA6 is the only individually essential player in driving the myocardial programme in *Xenopus*, the other two GATA factors are responsible for the residual expression of cardiac genes. Furthermore, in the absence of GATA6, their roles are increased. This is evident from their significantly greater effects on embryo phenotypes when combined with GATA6 MO compared to on their own.

GATA activity is required for differentiation but not induction of the myocardium in Xenopus

We have shown previously that GATA6 is required for the maintenance/maturation of the myocardium rather than its induction in both *Xenopus* and zebrafish embryos (Peterkin et al., 2003). As expected, based on the absence of a late phenotype, embryos injected with GATA4 or GATA5 MOs had no effect on early *Nkx2.5* expression, as seen for GATA6 MO (n=60, 72, and 89, respectively) (Fig. 3A). In order to determine if the lack of an early effect, even for GATA6 which has a strong late phenotype, was the result of redundancy within the GATA family, we examined *Nkx2.5* expression at neurula stages in embryos injected with all three MOs (n=102) (Fig. 3A). Expression was unaffected, as seen with each of the MOs on their own. Although *Nkx2.5* is also expressed in the underlying endoderm at this time, we showed by examining sections that the signal in the cardiac mesoderm is unaffected (Fig. 3B, territory delineated by dashed lines). Furthermore, a similar result was obtained for *Nkx2.3* (n=55) (Fig. 3C), which is not expressed or is very weak in the endoderm at this time (Fig. 3D). The expression of eHAND was also unaffected at this stage (Fig. 3E, territory delineated by dashed lines). We therefore conclude that, despite their earlier expression, GATA factors are not required for induction of the myocardial programme in *Xenopus*, as seen by the continued expression of the other early regulators, Nkx2.5, Nkx2.3 and eHAND, and their own continued expression, but rather for its maintenance/maturation.

GATA4 is not essential for induction or differentiation of zebrafish myocardium

GATA5 (*faust*) mutant zebrafish have profound defects in the myocardium, displaying reduced expression of several myocardial genes (Reiter et al., 1999). In addition, GATA6 has been shown to be required for maintenance/maturation of the myocardial programme in zebrafish as seen in *Xenopus* (Peterkin et al., 2003). In order to determine the relative effects of these two GATA factors, and to determine the contribution of GATA4, we separately injected into zebrafish embryos MOs
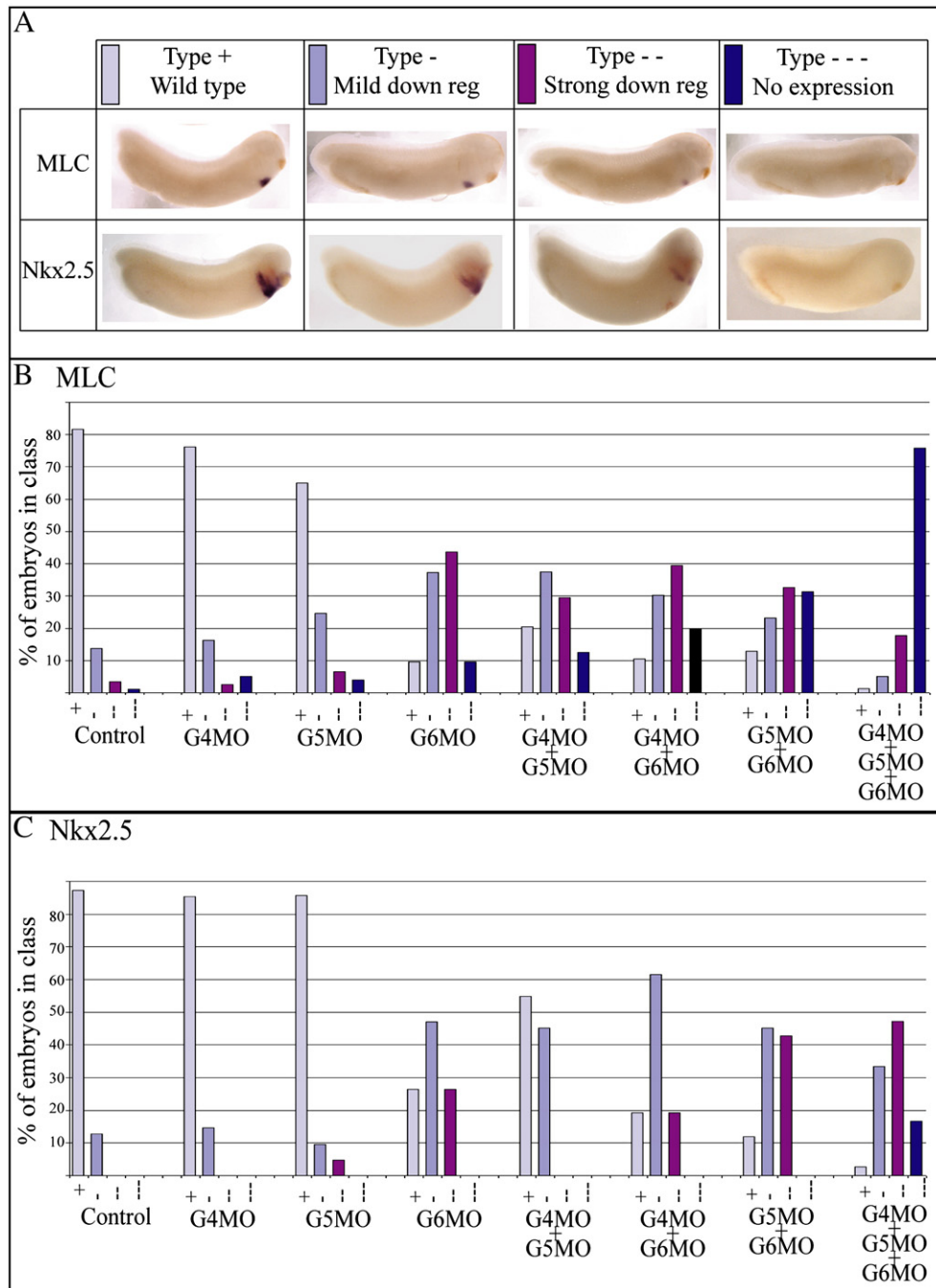
Fig. 2. Functional redundancy between GATA4, 5 and 6. (A) *Xenopus* embryos were injected singly and in combination with GATA4, 5 and/or 6 morpholinos, harvested at stage 28 and analysed by whole mount in situ hybridisation for *MLC* and *Nkx2.5* expression. Morphant embryos were classified into four classes: wild type (type +, light blue bar), mild down regulation of *MLC* or *Nkx2.5* (type −, mid blue bar), strong down regulation (type − −, purple bar) and no expression (type − − −, dark blue bar). (B, C) Graphical representations of the proportion of embryos in each class.

against each of these GATA factors. The GATA4 MO was shown to specifically block translation of a co-injected *GATA4* RNA and not *GATA5* or *GATA6* RNAs (Supplementary Figs. 1A, B, C). The GATA5 MO was designed to block splicing between exons 1 and 2 of the *GATA5* gene, which was confirmed in injected embryos by RT–PCR (Supplementary Figs. 1D, E). This splice blocking morpholino was designed upstream of the exons encoding the zinc fingers to prevent any protein produced binding DNA. However, the creation of a

dominant negative GATA5 via splicing from an upstream cryptic site is formally possible (see Supplementary Fig. 1D) but the ability of GATA4 and 6 morpholinos to enhance the cardiac phenotype in combinations (see below) makes this unlikely. Furthermore, the GATA5 morphant heart phenotype was indistinguishable from that seen in the *faust* mutant, both in single and combination experiments (Supplementary Fig. 1F and see below). The GATA6 MO has been reported previously (Peterkin et al., 2003).
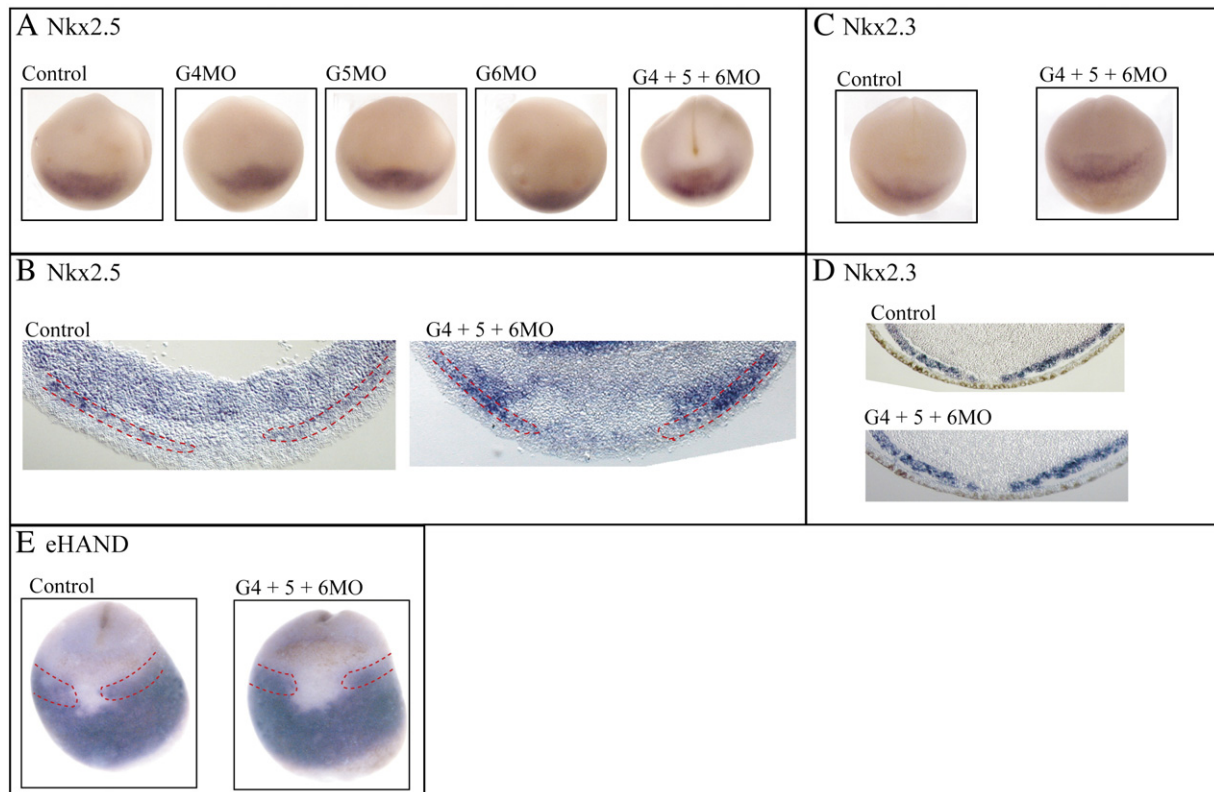
Fig. 3. Induction of cardiac precursor gene expression is unaffected in morphant *Xenopus* neurulae. (A) *Nkx2.5* was expressed normally at stage 16/17 when GATA4, 5 or 6 were depleted individually or in combination. (B) Cryostat sections confirmed that expression of *Nkx2.5* in the cardiac mesoderm (delineated by red dashed lines) was not affected. (C) Expression of *Nkx2.3*, which unlike *Nkx2.5* is restricted to the cardiac mesoderm (D) also remained unchanged in the triple morphants, as does *eHAND* (E). (Red dashed lines mark the cardiac precursors, remaining stain reflects expression in the blood island mesoderm).

The effects of the three MOs injected separately into zebrafish embryos were determined by monitoring expression of the transcription factor, *nkx2.5*, and the contractile machinery genes, *ventricular myosin heavy chain* (*vmhc*) and *cardiac myosin light chain 2* (*cmlc2*) (Fig. 4). GATA5 and 6 MOs induced cardia bifida as described previously for the *faust* mutant and the GATA6 MO (Peterkin et al., 2003; Reiter et al., 1999). In contrast, in GATA4 MO injected embryos, the myocardial cells appeared to have migrated and fused normally at the midline. We therefore conclude that, in zebrafish, only GATA5 and 6 are required for the proper migration of cardiac precursors. In contrast to mice and *Xenopus*, GATA4 appears to be uninvolved in this process.

The previously reported effects on myocardial gene expression of GATA5 or GATA6 knockdown (Peterkin et al., 2003; Reiter et al., 1999) were immediately evident in these MO injected embryos (Fig. 4). GATA5 MO injection led to substantially reduced expression of *nkx2.5* (19/19), *vmhc* (42/43) and *cmlc2* (40/41), as seen for the *faust* mutant. GATA6

MO injection also resulted in reduced expression of these markers (6/6, 60/60 and 42/44) but to a lesser extent. In contrast, GATA4 MO injection had little or no effect on cardiac marker gene expression levels ($n=28$, 69 and 53). Spatially the expression of the markers in the GATA4 morphants looks altered compared with the controls due to defects in late cardiac morphogenesis, consistent with those described by Holtzinger and Evans (2005). We therefore conclude that for laying down the myocardial programme in zebrafish, GATA5 has the greatest effect with a significant contribution from GATA6. In contrast, GATA4 makes little or no contribution, at least to the expression of the markers tested.

GATA factor redundancy in zebrafish myocardium

To determine if, as in *Xenopus*, there is redundancy within the GATA family in zebrafish, we injected the MOs in combinations (Fig. 4). Morphant embryos were classified into three types, unaffected (type +), down regulated (type −) or

Fig. 4. GATA5 and 6 are essential but GATA4 is redundant in zebrafish myocardium. Zebrafish embryos were injected with GATA4, 5 and/or 6 morpholinos individually or in combination and analysed by whole mount in situ hybridisation at 26 hpf for the expression of *Nkx2.5* (A, B), *vmhc* (C, D) and *cmlc2* (E, F). Anterior views: red brackets and arrowheads identify the cardiac expression. Embryos were classified into unaffected (type +), downregulated (type −) and absent (type − −) for expression of *nkx2.5* (B), *vmhc* (D) and *cmlc2* (F) and graphically represented. The effect on all three cardiac genes studied was similar for each injection. Depletion of GATA4 alone had little impact whereas loss of GATA5 or 6 caused a reduction in the expression of all three. Combinatorial ablation of GATA4+5 and GATA4+6 caused a further reduction in expression compared to the ablations of GATA5 or GATA6 alone thus demonstrating functional redundancy for GATA4 in the expression of these cardiac genes. GATA5+6 ablation resulted in complete loss of expression demonstrating an additive effect for these two GATAs.
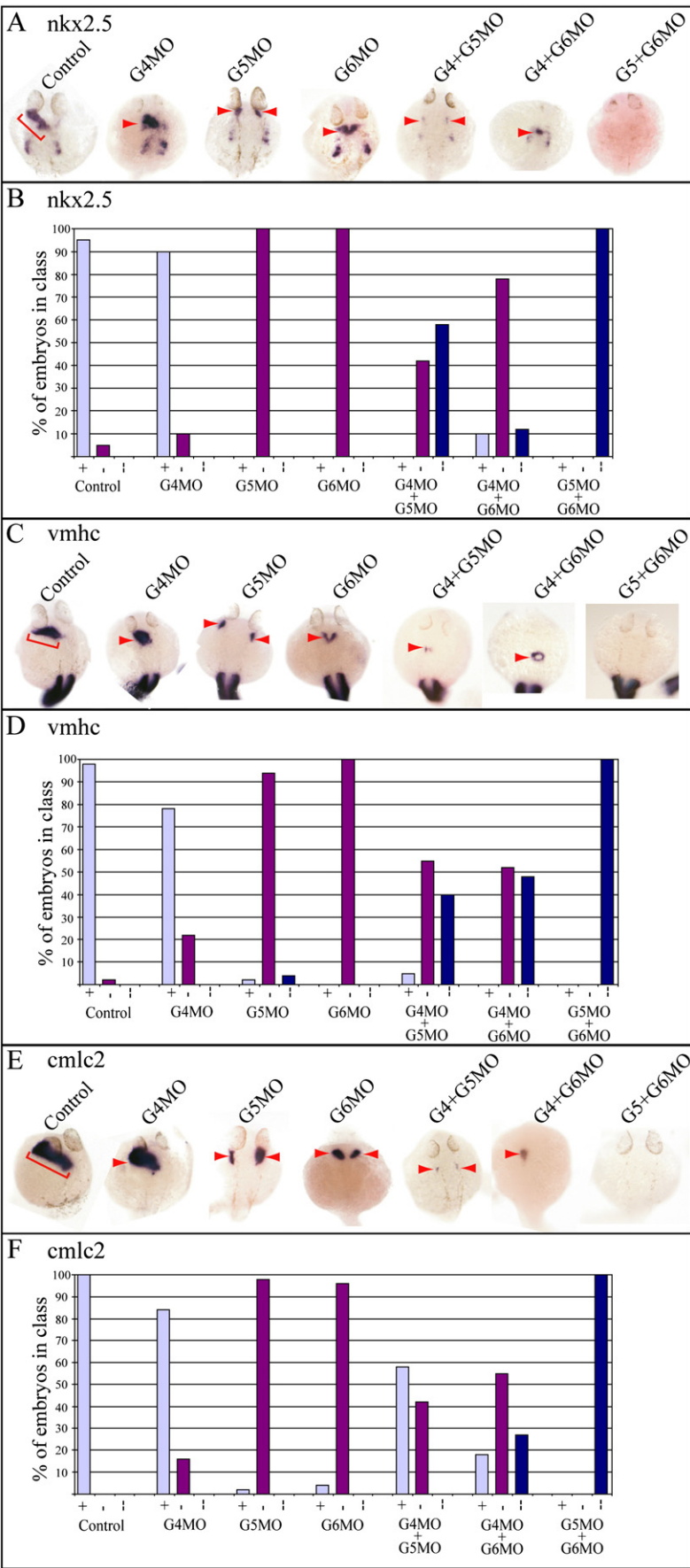
absent (type −−). Both the GATA5 and the GATA6 MO phenotypes were made worse by the co-injection of the GATA4 MO (Figs. 4B, D and F), as seen in *Xenopus*, and despite the fact that the GATA4 MO had little or no effect when injected on its own (*n* = 18–39). We therefore conclude that a significant proportion of the residual cardiac gene expression in GATA5 or GATA6 MO injected embryos is driven by GATA4, even though the consequences of its loss in the presence of GATA5 or GATA6 are minimal. Thus, redundancy within the GATA family is apparent in the zebrafish myocardium as in *Xenopus*.

The level of residual cardiac marker expression in the GATA4 and 5 MO combination or the GATA4 and 6 MO combination at 26 hpf was very low (Fig. 4). The level for the GATA5 and GATA6 MO combination was undetectable with 100% of the embryos losing expression, suggesting that, while GATA4 can cover for the absence of either GATA5 or GATA6, it cannot cover for the absence of both, which seems unlikely. We therefore monitored the expression of *GATA4* in flat-mounted (Fig. 5A) MO injected 10-somite embryos to determine if it was still expressed (Fig. 5C). We found that GATA5 MO on its own caused a reduction in *GATA4* expression (22/36 embryos), and residual expression was removed completely by the addition of the GATA6 MO (*n* = 35) (Fig. 5C). The *GATA4* expression seen in GATA4 morphants reflects the use of a translation-blocking morpholino rather than a splice-blocker. In the same experiment the expression of *nkx2.5* was affected in the same way as already described (Fig. 5B). We therefore conclude that the complete absence of cardiac marker expression in GATA5 plus GATA6 MO injected embryos results from the simultaneous absence of *GATA4* expression. Thus, as

seen for *Xenopus* embryos, the absence of all three GATA factors completely abolished cardiac marker expression.

GATA activity is required for induction of the myocardial programme in zebrafish

We have shown that GATA activity is only required for the maintenance/maturation of the myocardial programme in *Xenopus*. While we have shown that the GATA6 requirement in zebrafish is also late (Peterkin et al., 2003), *nkx2.5* expression at 6 somites has been shown to be affected in zebrafish *faust* mutants (Reiter et al., 1999), suggesting that an additional difference between the species might be the timing of requirements for GATA activity. We therefore tested this earlier requirement with more markers and to determine if it is subject to redundancy. Firstly, we examined *nkx2.5* expression in MO injected embryos at 5 somites when it is first expressed (Fig. 6A). For the GATA5 MO, we found a major reduction in expression totally consistent with the reductions seen later and with those reported for the *faust* mutant (Reiter et al., 1999). We also observed very little effect for the GATA4 or 6 MOs on their own, but both made the GATA5 MO phenotype more severe, consistent with their back-up roles being active at this early stage. Similar observations were made for *GATA4* and *hrt* expression in 5-somite embryos and for *tbx5* expression in 10-somite embryos (*tbx5* expression is first detected at ∼7 somites) (Figs. 6B, C, D). In contrast, *nkx2.7* expression was unaffected even by triple knockdown (Fig. 6E). We therefore conclude that, for the markers studied and in contrast to *Xenopus*, establishing the full early myocardial programme in zebrafish depends on GATA activity. The continued presence of cells expressing
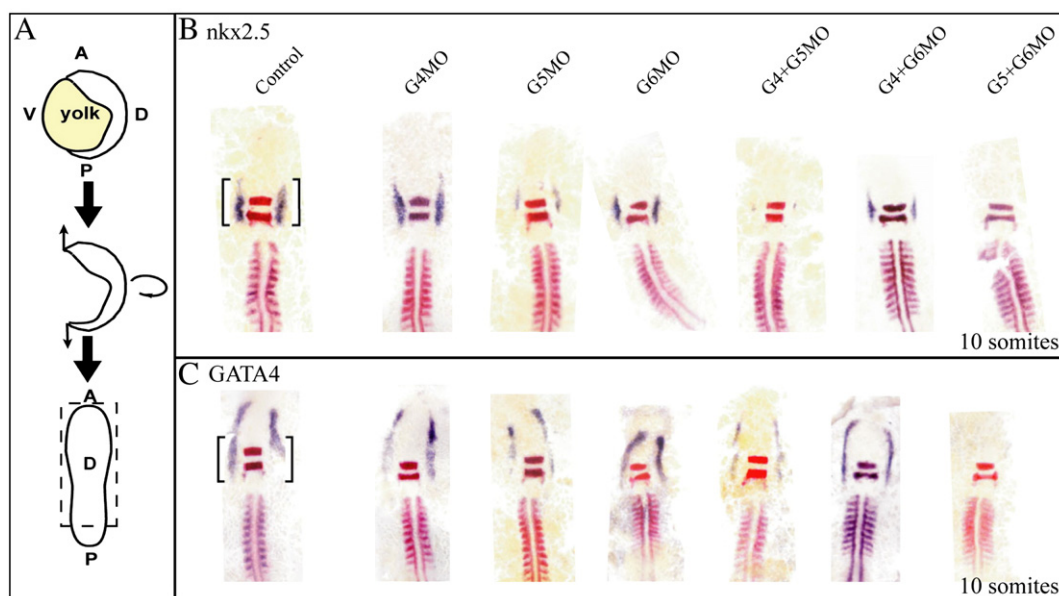

Fig. 5. Expression of *nkx2.5* and *GATA4* in zebrafish morphants of GATA4, 5 and/or 6 separately and in combination. Flat mounts, dorsal views (A) A, anterior; P, posterior; D, dorsal; V, ventral. (B) As seen at 26 hpf (Fig. 4), GATA4 morphants at 10 somites showed normal expression of *nkx2.5*, whereas GATA6, and more severely GATA5, morphants showed a reduction in expression. In combination morpholino injections, down regulation was exacerbated in each case, as seen at 26 hpf (Fig. 4), with expression being completely absent in embryos depleted for both GATA5+6. (C) Loss of GATA5 reduces the expression of *GATA4* and there is a complete loss of *GATA4* expression when GATA5 and 6 are depleted in combination (G5+G6MO). The black brackets indicate the cardiac expression. *Krox20* and *MyoD* are stained in red and were used as landmarks. *Krox20* is expressed in rhombomeres 3 and 5, *MyoD* was used to confirm the number of somites in the embryo.
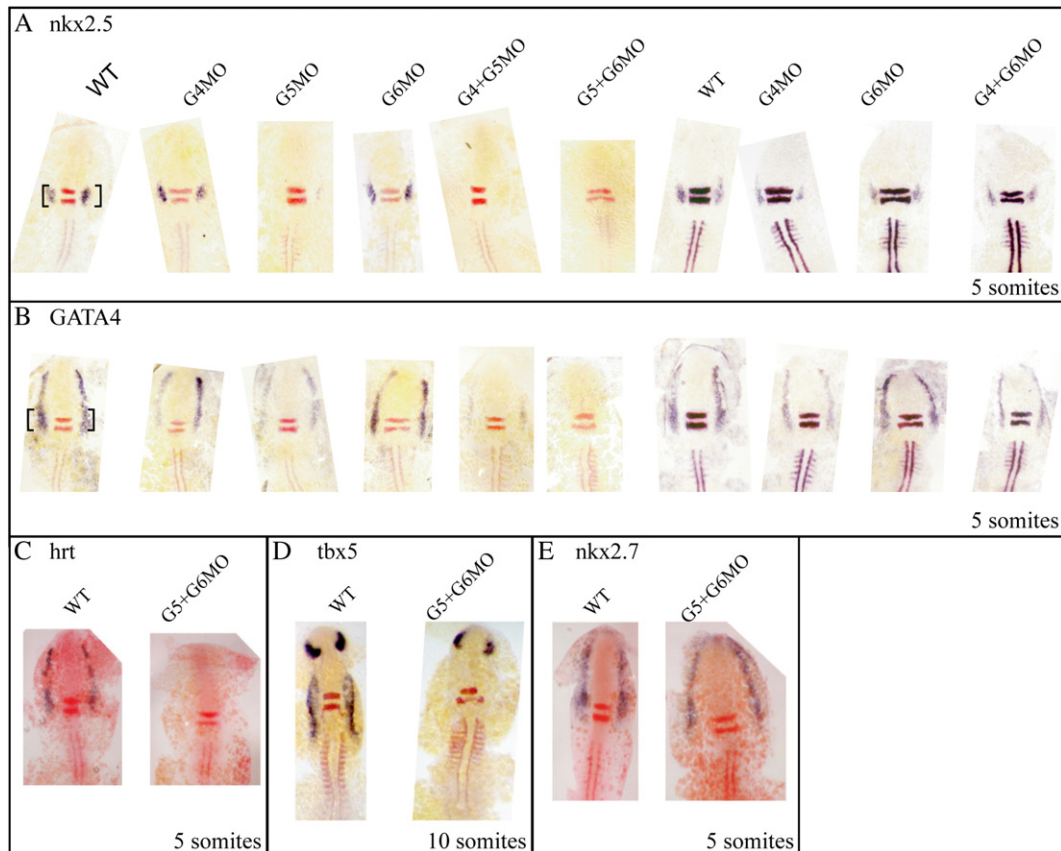
Fig. 6. Cardiac induction requires GATA activity in zebrafish. Flat mounts: dorsal views. (A) *nkx2.5* expression at 5 somites requires GATA5 and redundantly GATA4 and GATA6. The loss of GATA4 and/or GATA6 alone has little effect on the initiation of *nkx2.5*. (B) *GATA4* expression at 5 somites requires GATA5 and redundantly GATA6. GATA4 is also required to maintain its own expression (B). *Hrt/tbx20* expression is lost in the GATA5 and 6 double morphants (C) as is Tbx5 expression at 10 somites (D). (E) *nkx2.7* expression remains unchanged in embryos injected with GATA5 and 6 morpholino. Note that GATA5 and 6 double morphants knock out GATA4 expression and therefore represent a triple knockdown. The black bracket indicates the cardiac expression.

nkx2.7 suggested that apoptosis had not yet occurred, and this was confirmed by TUNEL and acridine orange assays (data not shown). Furthermore, re-specification to more anterior or more posterior mesodermal fates was not observed, as judged by the domains of expression of anterior lateral plate and pronephric markers (data not shown). We therefore conclude that in the absence of GATA activity, the cells remain undifferentiated at least up to the 10-somite stage.

Discussion

Redundancy

GATA4, *5* and *6* are an example of a gene family co-expressed in a specific tissue, in this case the myocardium. Although some differences in their binding site preferences have been detected (Sakai et al., 1998), all three bind to canonical GATA sites with high affinity. Because of this and the relatively mild phenotypes generated in loss of function experiments, they have been suggested to act redundantly (Jiang et al., 1998; Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1996; Watt et al., 2004). Here for the first time we present evidence in support of this with respect to laying down the genetic programme of the myocardium. The redundancy is

particularly striking for GATA4, whose individual loss has essentially no effect on induction or maturation of the myocardium in either zebrafish or *Xenopus*, in contrast to assumptions of its importance in much of the literature. For this member of the family, its contribution is only revealed in the absence of GATA5 or 6, thereby constituting a formal demonstration of redundancy. Similar demonstrations are evident for both *Xenopus* GATA5 and in early heart induction for zebrafish GATA6, where they are not the essential players. These redundant GATA activities thus most likely account for the residual expression of cardiac markers in the absence of the essential GATA factor. Indeed little change was observed in expression of the remaining GATA factor in double morphant embryos compared to wild type siblings in either zebrafish or *Xenopus* (data not shown). The one exception was GATA5 and 6 double morphant zebrafish embryos where the complete loss of GATA4 was used to effect a triple knockout (Fig. 5C).

GATA factors are an ancient family and in vertebrates have existed with three family members in the heart at least since fish (Patient and McGhee, 2002). Thus, the redundancy reported here would appear to be evolutionarily very stable. Maynard Smith and colleagues have developed simple genetic models to analyse selection pressures on redundant genes and have concluded that evolutionary stability can be achieved if the

two (or more) genes perform the same function, but with slightly different efficacies, as seen here (Nowak et al., 1997). The less efficient family member comes into its own when paired with a mutant form of the more efficient family member. Another evolutionarily stable model can be achieved where two (or more) genes perform more than one function: the redundancy occurring only with respect to one specific function. GATA4, 5 and 6 have an ever-growing list of functions in other tissues, so this scenario is more than adequately satisfied as well (Afouda et al., 2005; Capo-Chichi et al., 2005; Ketola et al., 2004; Molkentin, 2000; Yang et al., 2002). The evolutionary stability of this model depends on random mutations being more likely to render the genes inactive for all functions rather than just for one of their functions. Finally, yet another model suggests that redundancy should be more common in genes displaying specific spatio-temporal expression patterns during development, as is the case for GATA4, 5 and 6. For this model, the developmental error rates applicable to these genes need to be higher than their germ line mutation rates: a requirement that is currently unknown.

The primary GATA factor

An unexpected finding was that the member of the family whose loss has the biggest effect differs between *Xenopus* and zebrafish. For single knock downs, GATA6 has the strongest effect on myocardial gene expression in *Xenopus* whereas GATA5 does so in zebrafish. Although at first glance this might suggest a switch in roles for GATA5 and 6, a consideration of the timing of their actions suggests an alternative view. The action of GATA6 in *Xenopus* is after the initial expression of other early markers such as *Nkx2.5*, suggesting a role in differentiation of the myocardium (Peterkin et al., 2003). GATA6 knockdown in zebrafish has a very similar effect. Thus, in both organisms, knockdown of GATA6 leaves early marker expression initially intact but decaying with time, whereas when GATA5 was knocked down in zebrafish, expression of *Nkx2.5* and other early markers was compromised from the outset (Reiter et al., 1999; this study). The difference between the two organisms therefore can be characterised as the gain or loss of an early function for GATA5. The early role for GATA activity in zebrafish appears not to be masked by redundancy in *Xenopus* because even triple knockdown of GATA4, 5 and 6 leaves early expression of myocardial markers intact.

The role of GATA5 in myocardial induction in mouse and chick embryos is currently unclear. Although in P19 embryonal carcinoma cells induced to differentiate into cardiomyocytes, GATA5 up-regulation occurs after Nkx2.5, precluding an early function during induction (Alexandrovich et al., 2006), the mouse knockout of *GATA5* retained the capacity to synthesise a truncated form of the protein containing both zinc fingers, which would likely have significant activity, preventing a definitive conclusion (Nemer and Nemer, 2002). Likewise, the attempts to date to knock down GATA5 activity in the chick were only partial and, in addition, attempted after induction of the myocardium (Jiang et al., 1998). It is therefore not yet clear if the early role for GATA5 has been acquired by zebrafish or

lost by *Xenopus*. In *Drosophila*, the GATA factor, *pannier*, is required both upstream and downstream of the *Nkx2.5* homologue, *tinman* (Gajewski et al., 2001; Klinedinst and Bodmer, 2003). In the nematode, the GATA factors, *Med1* and *2*, are expressed in the mesendodermal precursor to the mesoderm giving rise to part of the pharynx, an organ that has homologies to the heart, and upstream of the *Nkx2.5* homologue, *ceh22*, suggesting that an early role for GATA factors may be ancestral (Broitman-Maduro et al., 2006; Maduro et al., 2001; Rodaway and Patient, 2001). Mesendodermal expression is seen for both *GATA5* and *GATA6* in zebrafish, while in *Xenopus*, mesendodermal expression is seen for *GATA4* and *6* (Fletcher et al., in press; Rodaway et al., 1999; J. Broadbent, A. Gibson and R. Patient, unpublished observations). Thus, the early role for GATA5 in zebrafish may reflect its early expression in the lineage of cells leading to the myocardium whereas, in *Xenopus*, early expression of *GATA5* is restricted to the endoderm, appearing in the cardiac mesoderm at a later stage (Weber et al., 2000). That *GATA6* is also expressed early in this lineage in both species, and *GATA4* likewise in *Xenopus*, and yet neither plays a role in induction of the myocardium, suggests that GATA5, at least in zebrafish, is alone in containing the requisite amino acid sequence for this function. All three GATA factors recognise the same DNA sequence therefore, in the absence of known binding site preferences for GATA5, activities specific to GATA5 are likely to include protein–protein interactions. Thus, GATA5 may be more suited to the necessary interactions in the early mesendoderm than either GATA4 or 6.

Feedback loops and timing

Genetic regulatory networks (GRNs) consist of functionally linked regulatory genes encoding transcription factors and their controlling extra-cellular signals (Davidson et al., 2002). They contain motifs, or recurring wiring patterns, which occur with frequencies far greater than in randomised networks (Lee et al., 2002; Milo et al., 2002; Shen-Orr et al., 2002). One such motif is a feedback loop. Mathematical modelling of positive feedback loops indicate that they promote the persistence of signals and have the potential to store information, such that, for example, signalling can readily flip the system from one state to another (Bhalla and Iyengar, 1999). The observation in *Drosophila* that *pannier* is both upstream and downstream of *tinman*, raises the possibility that the establishment of a mutual regulatory loop for these two key regulators is critical evolutionarily (Gajewski et al., 2001; Klinedinst and Bodmer, 2003). Evidence for a similar feedback loop exists in mice, where a cardiac GATA gene has been shown to be Nkx dependent and vice versa (Brewer et al., 2005; Davis et al., 2000; Lien et al., 1999; Molkentin et al., 2000). Davidson and Erwin have recently proposed that regulatory motifs of this type are evolutionarily stable components of GRNs, which they called kernels (Davidson and Erwin, 2006). They highlight a heart-field specification kernel, which is conserved from *Drosophila* to vertebrates. Strikingly the *Nkx2.5/tinman GATA/ pannier* feedback loop is central to this kernel. Our work
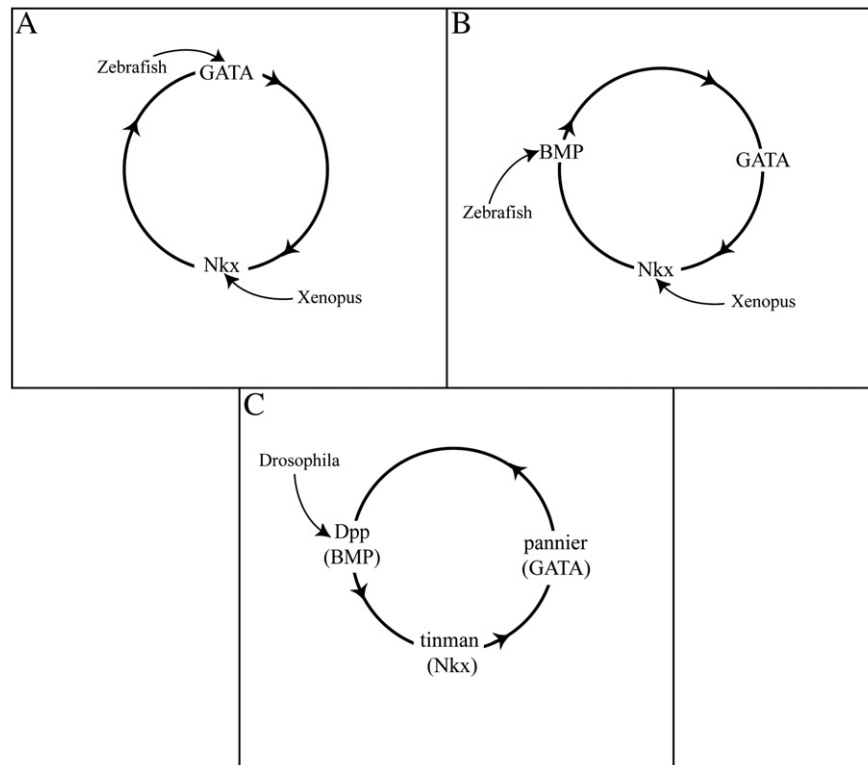
Fig. 7. GATA, Nkx and BMP regulation during cardiogenesis. (A) GATA and Nkx regulate each other in a positive feedback loop, which is initiated differentially in *Xenopus* and zebrafish. (B) BMP expression is regulated by and regulates the GATA/Nkx positive feedback loop. (C) The BMP/GATA/Nkx feedback loop is conserved in *Drosophila*, although the direction of flow is reversed and the maintenance of the loop requires expression of pannier and Dpp in the ectoderm (see Discussion).

supports this hypothesis and further suggests that the establishment of this kernel is more critical in evolution than where the loop is initiated. Thus, GATA activity is required to initiate *Nkx2.5* expression in zebrafish but not in *Xenopus*, nevertheless both establish the loop (Fig. 7A).

Interestingly, as seen for GATA activity, the requirement for BMP signalling differs between zebrafish and *Xenopus*. Thus, in zebrafish, BMP signalling is required to initiate expression of cardiac markers including *GATA5* (Reiter et al., 2001), whereas in *Xenopus*, it is only required for their maintenance (Walters et al., 2001). In view of the links between BMP and GATA factors in several different tissues, including the myocardium, it seems likely that the early requirement for GATA activity in zebrafish is linked to the early requirement for BMP, and likewise the later requirement in *Xenopus* (Fig. 7B) (Peterkin, 2003). The cascade of events in *Drosophila* predicts that the *Drosophila* BMP signal, Decapentaplegic (Dpp), activates *tinman*, and they then act in concert to initiate the expression of *pannier*. Subsequently *tinman* and *pannier* maintain each other's expression, whilst *pannier* (in the ectoderm) maintains *Dpp* expression. Dpp signalling feeds back to maintain expression of *tinman* and *pannier* thus completing the loop (Fig. 7C; for review see Sorrentino et al., 2005). Thus, in summary, the data imply that the initiating factor and the direction in which the loop flows are not important. Ultimately it is the establishment of the loop that is essential and failure to do so leads to the loss of differentiated myocardium.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.08.018.

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