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Induction of cardiomyocytes by GATA4 in *Xenopus* ectodermal explants

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SUMMARY

The earliest step in heart formation in vertebrates occurs during gastrulation, when cardiac tissue is specified. Dorsoanterior endoderm is thought to provide a signal that induces adjacent mesodermal cells to adopt a cardiac fate. However, the nature of this signalling and the precise role of endoderm are unknown because of the close proximity and interdependence of mesoderm and endoderm during gastrulation. To better define the molecular events that underlie cardiac induction, we have sought to develop a simple means of inducing cardiac tissue. We show that the transcription factor GATA4, which has been implicated in regulating cardiac gene expression, is sufficient to induce cardiac differentiation in *Xenopus* embryonic ectoderm (animal pole) explants, frequently resulting in beating tissue. Lineage labelling experiments demonstrate that GATA4 can trigger cardiac differentiation not only in cells in which it is present, but also in neighbouring cells. Surprisingly, cardiac differentiation can occur without any stable differentiation of anterior endoderm and is in fact enhanced under conditions in which endoderm formation is inhibited. Remarkably, cardiac tissue is formed even when GATA4 activity is delayed until long after explants have commenced differentiation into epidermal tissue. These findings provide a simple assay system for cardiac induction that may allow elucidation of pathways leading to cardiac differentiation. Better knowledge of the pathways governing this process may help develop procedures for efficient generation of cardiomyocytes from pluripotent stem cells.

Movies available on-line

Key words: GATA4, *Xenopus*, Cardiac induction, Heart, Endoderm

INTRODUCTION

In all vertebrates, heart formation begins very early in embryogenesis and apparently proceeds in a similar manner (Harvey, 2002). From a combination of embryological experiments and lineage labelling studies, we know that the embryonic heart is derived primarily from symmetrical, bilateral patches of mesodermal tissue that form during gastrulation. These converge at the embryo midline, fusing to form a linear contractile tube. While the final structure of the mature heart varies widely between different vertebrate classes, in each case transformation of the linear tube into a multi-chambered organ involves complex morphogenetic changes that are coordinated with regional differentiation of distinct cardiac cell types (Harvey, 2002).

Identifying the molecular events that specify cardiac fate and regulate orderly differentiation of cardiac tissues is essential for our understanding of congenital heart disorders, and may allow the development of novel therapies for cardiac disease. Although considerable progress has been made in identifying genes expressed in different regions of the developing heart and the mechanisms that regulate their transcription (Bruneau, 2002), our understanding of the key events that direct embryonic blastomeres to a cardiac fate and ultimately trigger the onset of terminal differentiation remains fragmentary. One reason for this is the lack of molecular markers uniquely associated with cardiac fate that would allow cardiac progenitors to be traced from the time of their specification until they initiate terminal differentiation. Another is the difficulty inherent in attempting to identify the cell-to-cell interactions necessary for the formation of cardiac progenitors in the complex and rapidly changing environment of the gastrulating embryo. Identifying the signalling pathways that mediate such interactions is further complicated by the findings that such pathways frequently have multiple roles in the early embryo that are difficult to distinguish.

Many studies have established the importance of endoderm in heart formation, establishing an important role for this tissue in facilitating normal cardiac morphogenesis. Several have also provided evidence for a distinct and much earlier role for endodermal tissue in the initial specification of cardiac progenitors. In amphibian and chick embryos, efficient formation of cardiac progenitors depends on an interaction between prospective cardiac mesoderm and the underlying anterior endoderm with which it is intimately associated (Lough and Sugi, 2000; Nascone and Mercola, 1995). Whether such interactions are essential for specifying cardiac cell fate in all vertebrates is less clear. For example, in the zebrafish, the *casanova* (*cas*) mutation inactivates a *Sox*-related transcription factor, resulting in a defect in endoderm formation (Kikuchi et al., 2001). Although heart morphogenesis is severely disrupted in these embryos, cardiac tissue is still formed, indicating that

cardiac specification occurs in the absence of *cas*-dependent endoderm (Alexander et al., 1999).

Molecular mediators of the cardiogenic inducing signal(s) have not yet been identified, but some candidate molecules have been proposed. In the chick embryo, bone morphogenetic proteins (BMPs) have been shown to be necessary for cardiogenic activity of anterior endoderm, and inhibitors of BMP activity suppress cardiac differentiation (Schlange et al., 2000; Schultheiss et al., 1997). In other vertebrates, evidence of a similar role for BMPs is less clear cut. In the mouse, homozygous null mutants for components of the BMP signalling pathway have not proved to be very informative because the phenotypes are either lethal prior to the onset of cardiogenesis or result in disruption of heart morphogenesis rather than absence of cardiac tissue (reviewed by Schneider et al., 2003). In the zebrafish, Bmp2 (Bmp2a – Zebrafish Information Network) mutant embryos show profound defects in many tissues, including the myocardium (Mullins et al., 1996; Reiter et al., 2001). The cardiac phenotype may indicate a specific role for Bmp2 in the specification of heart progenitors, but it may also be a consequence of earlier general dorsalisation of the entire embryo (Mullins et al., 1996). In *Xenopus* embryos, interfering with BMP signalling during gastrulation also produces a dorsalised phenotype (Dale and Jones, 1999). Restricting inhibition of the BMP pathway to later stages of development, or to lineages that include cardiac progenitors, has no effect on the heart field formation but results in reduction of differentiated cardiac muscle and disruption of heart morphogenesis (Breckenridge et al., 2001; Shi et al., 2000; Walters et al., 2001).

If BMP signalling is important for cardiac specification, there is also evidence that other signalling pathways are necessary to restrict the location of cardiac progenitors. In explants from chick and frog embryos, secreted antagonists of the WNT/β-catenin pathway, DKK1 and Crescent, promote cardiogenesis in posterior or ventral mesoderm, respectively (Marvin et al., 2001; Schneider and Mercola, 2001). This has led to the proposal that during normal development, expression of these antagonists in the organizer creates a zone of low WNT signalling in the adjacent pre-cardiac mesoderm, thereby delineating bilateral regions of anterior mesoderm capable of responding to an endoderm-derived cardiogenic signal (Schneider and Mercola, 2001).

Cardiac progenitors express the homeobox gene *Nkx2.5* soon after they have been specified. Mouse mutants that are homozygous for a null mutation of *Nkx2.5* show severe and early disruption in heart tube morphogenesis (Biben and Harvey, 1997; Tanaka et al., 1999), and defects in heart valve and septal development have also been associated with *Nkx2.5* mutations in humans (Benson et al., 1999; Schott et al., 1998). These results indicate roles for *Nkx2.5* both early and late in vertebrate cardiogenesis but further definition of these has remained elusive. The existence of multiple related, and perhaps functionally redundant, NKX2 family members in vertebrates has complicated the interpretation of mutant phenotypes. Additionally, *Nkx2.5* is expressed in other tissues of the early embryo (most notably in the anterior pharyngeal endoderm), and within the mesoderm it is unclear whether its expression identifies definitive cardiac progenitors or a broader domain of cells that can be diverted to a cardiac fate. Loss-of-function studies in *Xenopus* (Evans, 1999) and analysis of heart mutants in zebrafish confirms the importance of *Nkx2.5* expression for subsequent cardiac differentiation, and suggest that *Nkx2.5* lies downstream of a BMP-mediated cardiogenic signal (Reiter et al., 2001). However, in the absence of any specific marker of cardiac progenitor cells prior to the onset of terminal differentiation, it has proved difficult to identify the precise role of NK2 family members in the acquisition of cardiac cell fate.

Nkx2.5 does not appear to be a 'master regulator' of cardiac fate in the manner that members of the MYOD family drive skeletal muscle differentiation; indeed, no equivalent cardiomyogenic regulators have yet been identified. Instead, analysis of cardiac muscle-specific transcription has identified a number of transcription factors that, together and in multiple combinations, regulate cardiac transcription. These include proteins of the GATA, NK2 and MADS, and myocardin transcription factor families (Bruneau, 2002; Cripps and Olson, 2002), many of which are expressed in a broad range of tissues in the early embryo. Cardiac-specific transcription most likely results from the formation of particular multi-protein complexes by these factors within the differentiating cardiomyocyte (Bruneau, 2002; Cripps and Olson, 2002). Do these factors play any role establishing the population of cardiac progenitors prior to terminal differentiation? Once again, an unambiguous answer has been obscured by the expression of multiple members of each transcription factor family and the potential for functional redundancy between them.

Because of these difficulties, it would be advantageous to examine the ability of these factors to trigger cardiac differentiation in embryonic tissue that does not normally contribute to heart formation in the embryo. Furthermore, if such an assay can be conducted in cultured explants rather than the embryo, the contribution of the many cell-to-cell interactions and cell signalling events that occur during the critical period of gastrulation can be minimised. Here we show that such an assay system is provided by ectopic expression of GATA4 in animal pole explants from embryos of *Xenopus laevis*. Under these conditions, such presumptive ectodermal tissue reliably forms a restricted range of mesendodermal tissue derivatives, including cardiac muscle. We have used this assay to examine the competence of explants to undergo cardiac induction, the possible role of endodermal tissue within the explant and the role of signalling pathways proposed to mediate cardiac specification.

MATERIALS AND METHODS

Xenopus embryo culture

Xenopus embryos were obtained, injected and cultured in Normal Amphibian Medium (NAM) (Sive, 2000). Animal pole explants were excised at stage 8.5-9 and cultured in 0.75×NAM until the indicated stage. Dissociation of animal poles was carried out as previously described (Sive, 2000) (see Fig. 6). Dexamethasone (2 μM; Sigma), dissolved in ethanol, was added at the stages indicated. Cardiac actin-GFP transgenic frog lines have been described (Latinić et al., 2002).

RNA and DNA injections

Synthetic capped RNA was synthesised as described (Sive, 2000). The amounts injected were: 0.7-0.9 ng *Gata1*, *Gata4*, *Gata5*, *Gata6* (Jiang and Evans, 1996; Zhang and Evans, 1994) and *Gata4*-GR (R. Patient

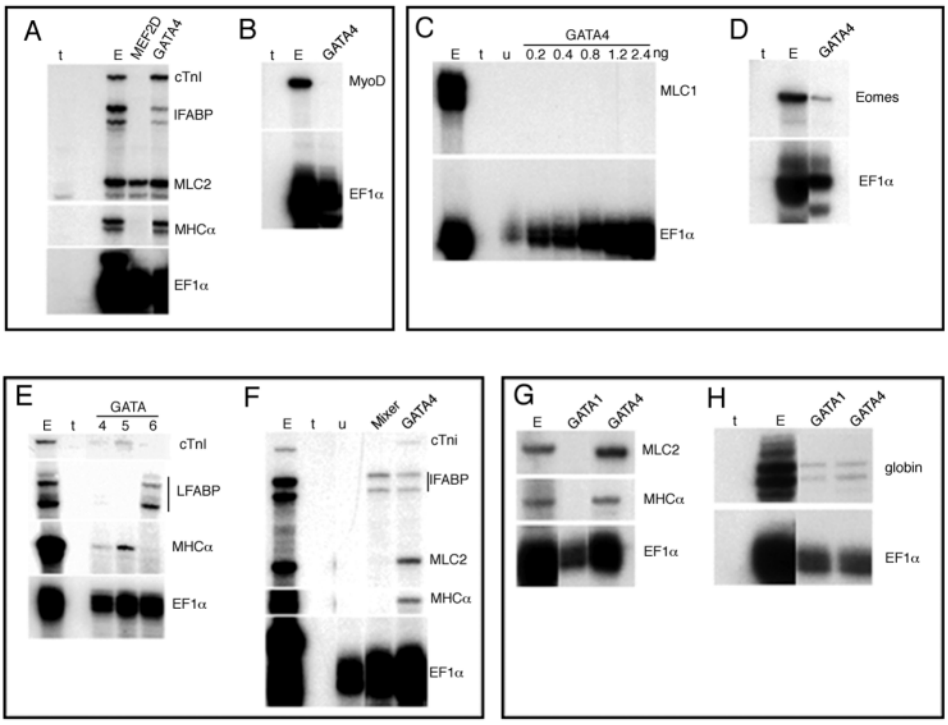

Fig. 1. GATA4 induces cardiac-specific genes in the absence of skeletal muscle. (A) RNase protection assay showing induction by GATA4 of three cardiac markers, as well as the gut marker IFABP (intestinal fatty acid binding protein). Note that MEF2D induces only MLC2 and not the complete cardiogenic programme. (B,C) Absence of myogenic factor MYOD at stage 18, as well as skeletal muscle marker MLC1 (myosin light chain 1), in *Gata4*-injected animal pole explants. A wide range of *Gata4* mRNA doses were injected to rule out concentration-dependent effect on MLC1 expression. (D) Eomesodermin is induced by GATA4 at stage 10.5. (E) GATA4 and GATA5 are efficient inducers of cardiac tissue, whereas GATA6 is more efficient in inducing LFABP (liver fatty acid binding protein). (F) Mixer and GATA4 induce gut tissue (IFABP), whereas only GATA4 additionally induces cardiac tissue. GATA4 induces IFABP in some, but not all experiments where cardiac markers were induced (data not shown). GATA1 does not induce cardiac tissue (G), whereas both GATA1 and GATA4 induce blood marker globin (H). Samples for RNA expression analysis were collected at stage 37/38. (EF1α: elongation factor 1 α; t: transfer RNA control; u: uninjected animal caps; E: embryo control.)

and B. Afouda, unpublished), unless indicated otherwise; 1 ng Mixer (Henry and Melton, 1998), *Cerberus* (Bouwmeester et al., 1996), *Dkk1* (Glinka et al., 1998), *Dsh ΔPDZ* (Sokol, 1996), *DEP+* (Tada and Smith, 2000), *Chordin* (Sasai et al., 1994); 0.2 ng *Sox17βEnR* (Hudson et al., 1997); 0.4 ng *Bmp4* (Dale et al., 1992); 0.1 ng *CSKA-XWnt8* DNA (Smith and Harland, 1991). The activity of all constructs used in epistasis experiments was confirmed by: observing the phenotype of injected embryos [SOX17βEnR (loss of endoderm) and CSKA-XWNT8 (posteriorisation)]; assaying for cement gland induction in animal caps (DKK1, Cerberus and Chordin); and blocking the activin-induced elongation of animal caps (dominant-negative Dishevelled constructs; data not shown). 10 pg each of TOPFLASH (van de Wetering et al., 1997) and RL-TK (Promega) reporter plasmids were injected, and luciferase assays were performed using the Dual Luciferase Assay kit (Promega). Progeny of injected blastomeres were visualised using rhodamine-dextran or by biotinylated-dextran lineage markers (Molecular Probes).

RNA analysis

RNA was isolated as described (Chomczynski and Sacchi, 1987). 15-20 animal caps were used per sample. The templates for riboprobes were: *MHCα* (Logan and Mohun, 1993), *MLC2* (Chambers et al., 1994), *cTnI* (Drysdale et al., 1994), *IFABP* (Shi and Hayes, 1994), *Sox17α* (Hudson et al., 1997), *MyoD* (Hopwood et al., 1989), *MLC1* (Theze et al., 1995), *Eomesodermin* (Ryan et al., 1996), *globin* (Patient et al., 1982), *Nkx2.3* (Evans et al., 1995), *Nkx2.5* (Tonissen et al., 1994), *For1* (Seo et al., 2002), *EF1α*, *amylase* and *insulin* (Horb and Slack, 2002). For the *LFABP* probe, we used the 5′ end of cDNA [derived by 5′ RACE using partial cDNA information; see Henry and Melton (Henry and Melton, 1998)]. The *Gata4* probe was derived from 3′UTR to distinguish it from the injected transcript. RNase protection assays were performed using a rapid hybridisation method (Mironov et al., 1995). Whole-mount in situ hybridisation (Sive, 2000) using digoxygenin-labelled *XMLC2* probe has previously been described (Chambers et al., 1994). Injected biotinylated dextran was revealed with ExtrAvidin-alkaline phosphatase (Sigma) using magenta-phos (Sive, 2000) as a substrate.

RESULTS

GATA4 induces cardiomyocytes in the absence of skeletal muscle

Using *Xenopus* embryo ectodermal explants, we examined the cardiac-inducing capacity of transcription factors implicated in heart development (GATA4, GATA5 and GATA6, NKX and MADS families) (Bruneau, 2002). We found that GATA4 is sufficient to induce expression of three cardiac muscle-specific differentiation markers: Myosin Light Chain 2 [MLC2 (Chambers et al., 1994)], Myosin Heavy Chain α [MHCα (Logan and Mohun, 1993)] and cardiac Troponin I [cTNI (Drysdale et al., 1994)] (Fig. 1A). These markers, which are entirely restricted to the myocardium, appeared in explants at the same time as in sibling embryos, suggesting that correct activation of the cardiac differentiation programme occurred (Fig. 6). In some cases, expression of GATA4 in animal pole explants caused formation of spontaneously beating tissue, confirming that functional cardiomyocyte differentiation had occurred (see Movie 1 at http://dev.biologists.org/supplemental/). Induction of beating cardiac tissue has previously been achieved in *Xenopus* animal pole explants by exposure to activin (Logan and Mohun, 1993) but, in addition to cardiac muscle, activin treatment induces skeletal muscle along with a wide range of other mesendodermal cell types (Green et al., 1992; Gurdon et al., 1996). By contrast, no skeletal muscle was formed in GATA4-expressing explants, as judged by the absence of the myogenic factor XMYOD (Fig. 1B) or the

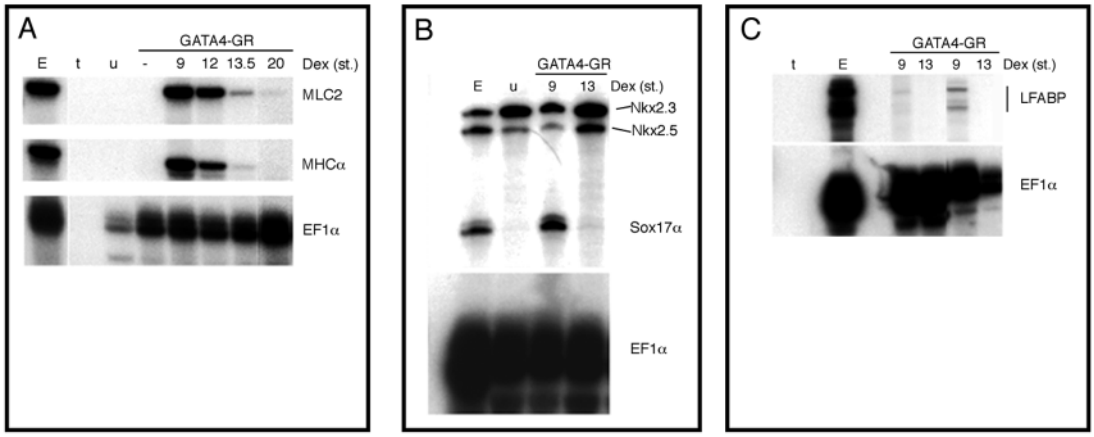

Fig. 2. Gradual loss of cardiogenic activity of GATA4. Cardiac tissue can be induced after gastrulation, and even during tailbud stages. (A) *GataA4*-GR was activated by the addition of dexamethasone (dex) at stage 9, 12, 13.5 or 20. (B) *Sox17α* is efficiently induced when *Gata4* is activated at stage 9, but is induced poorly when it is activated at stage 13. *Nkx2.5* and *Nkx2.3* are expressed in uninjected animal caps (see also Fig. 5). (C) *LFABP* is only induced if *Gata4*-GR is activated at stage 9, but not at stage 13. Two different experiments are shown, and an additional two yielded the same result. Analysis was performed at stage 37/8 (A,C) and stage 18 (B), respectively.

skeletal muscle differentiation marker MLC1 (Fig. 1C). This result suggests that GATA4 has a more restricted effect on explant cell fate than activin. Consistent with this, *Gata4*-injected explants expressed markers of dorsoanterior mesoderm (Eomesodermin; Fig. 1D) and endoderm [SOX17α (Weber et al., 2000)] and XHEX (data not shown), but not the early marker of posterior mesoderm, XBRA (Weber et al., 2000).

We tested the specificity of GATA4 in inducing cardiac tissue by comparing it with other members of the GATA family. GATA4, GATA5 and GATA6 have all previously been implicated in the development of embryonic heart, lung and gut tissue (Patient and McGhee, 2002), but it is not known whether this includes a role in the initial specification of cardiac cell fate. We found that, in our assay, GATA5 has similar cardiogenic activity to that of GATA4, while GATA6 was considerably less active (Fig. 1E). Interestingly, GATA6 was a more efficient inducer of liver tissue than GATA4 or GATA5, as judged using the liver-specific marker LFABP [Liver Fatty Acid Binding Protein (Henry and Melton, 1998); Fig. 1E]. We also tested the activity of GATA1, which is involved in regulating haematopoiesis (Huber and Zon, 1998). As previously reported, GATA1 induced the formation of blood in animal cap explants (Fig. 1H) (Mead et al., 2001), but was unable to induce any cardiac markers (Fig. 1G). Interestingly, GATA4 also induced the formation of blood marker globin (Fig. 1H).

Induction in explants can still occur after gastrulation

In normal development, induction of cardiac mesoderm is thought to occur rapidly during gastrulation of the embryo (Harvey, 2002). We employed a dexamethasone-inducible version of *Gata4* to investigate whether the window for triggering subsequent cardiac differentiation in explants is similarly restricted. By adding dexamethasone at progressively later stages of development, we found that cardiac tissue was still formed even when *Gata4* activation was delayed for several hours after explant isolation, until sibling embryos had reached neurula stage (Fig. 2A). In fact, a small amount of cardiac tissue was ultimately formed even when dexamethasone treatment was delayed until the equivalent of tailbud stages (stage 20-25; Fig. 2A and data not shown). As the competence of animal pole cells to respond to FGF and activin signaling is lost by the late gastrula stage (Gillespie et al., 1989; Grimm and Gurdon, 2002), this result suggests that GATA4 does not require these functional signalling pathways for induction of cardiac tissue. Importantly, explants that are converted to a cardiac fate by such a delayed activation of GATA4 have already undergone epidermal differentiation (Jonas et al., 1985). GATA4 presumably triggers cardiogenesis in these explants either through the transdifferentiation of epidermal tissue, or by acting on a remaining population of pluripotent stem-like cells.

SOX17-dependent endoderm antagonises cardiogenesis by GATA4

In normal amphibian development, signals from endoderm are believed to induce cardiac fate in adjacent mesoderm. Endoderm induction in explants is not by itself sufficient to trigger cardiac muscle formation, as ectopic expression of the endoderm inducer Mixer (Henry and Melton, 1998) resulted in the expression of gut endoderm markers but no cardiac markers were activated (Fig. 1F). Ectopic GATA4 also induces endodermal tissue in animal pole explants (Weber et al., 2000) (Fig. 1F), and to test whether the cardiogenic activity of GATA4 requires the presence of such induced endodermal tissue, we injected *Gata4* together with an inhibitor of endoderm formation.

Sox17α and *β* are key regulators of early endoderm development (Hudson et al., 1997), and expression of the dominant negative construct *Sox17βEnR* (which blocks both *Sox17α* and *β*) inhibits early endoderm development in *Xenopus* embryos and explants (Hudson et al., 1997). Surprisingly, co-injection of *Sox17βEnR* with *Gata4* led to a substantial increase in cardiac tissue formation (Fig. 3B) and severe reduction in the formation of anterior endodermal tissue, such as liver, as judged by LFABP (Fig. 3F) and For1 (Seo et al., 2002) (data not shown). The apparent synergism between SOX17βEnR and GATA4 in promoting cardiac tissue differentiation suggests that GATA4 may induce endoderm at

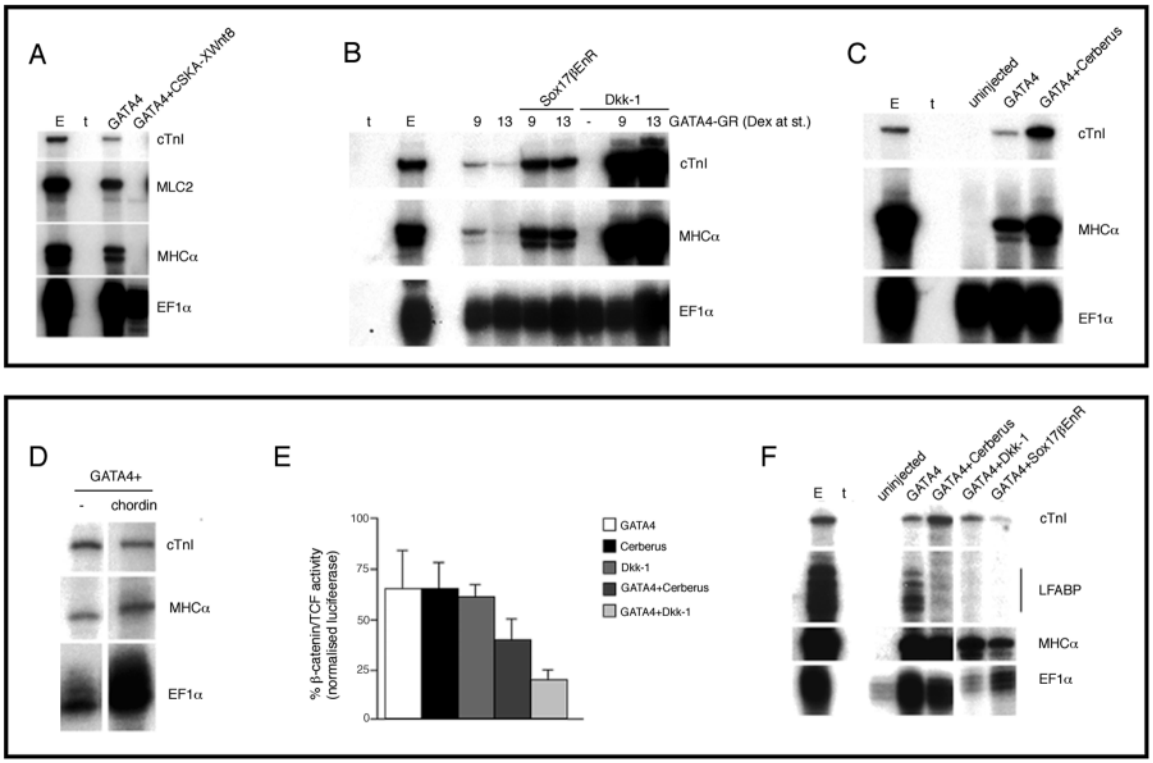

Fig. 3. SOX17-dependent endoderm and canonical WNT signalling antagonise GATA4. (A) Expression of XWNT8 from the cytoskeletal actin promoter (CSKA) completely inhibits GATA4-mediated cardiogenesis. (B) SOX17βEnR and the WNT antagonist DKK1 greatly synergise with GATA4 when the latter is activated either at stage 9 or at stage 13. (C) Cerberus, a tri-valent inhibitor of Nodal, BMP and WNT ligands also synergises with GATA4. (D) Chordin does not inhibit GATA4-mediated cardiac tissue formation, confirming that BMP is not required. (E) DKK1, Cerberus and GATA4 antagonise β-catenin/TCF-dependent transcription in animal caps. The TOPFLASH reporter was co-injected with internal control TK-Renilla luciferase together with indicated RNA. Animal caps were collected at stage 13, and baseline level of normalised luciferase activity (TOPFLASH alone) was set at 100%. Data from three experiments were used. (F) No LFABP can be detected under conditions of efficient cardiac tissue induction. All samples were analysed for RNA expression at stage 37/8.

the expense of cardiac tissue. Consistent with this, other treatments that enhanced the extent of cardiac differentiation in GATA4-expressing explants, such as co-expression of DKK1 and Cerberus (see below), also resulted in a profound reduction or complete absence of liver differentiation (Fig. 3F). Markers for other anterior endodermal tissues, such as pancreas [e.g. insulin and amylase transcripts (Horb and Slack, 2002)], were also undetectable (data not shown).

Together these results indicate that, at least in GATA4-induced explants, differentiation of endoderm and cardiac muscle tissue are mutually antagonistic, and that stable endoderm formation is not essential for cardiac tissue induction. Further support for this interpretation is provided by results from the explants expressing the hormone-inducible form of GATA4. If GATA4 activity is delayed until the equivalent of early neurula stage (stage 13), expression of the early endodermal marker *Sox17α* is greatly reduced (Fig. 2B). Under such conditions, no markers for anterior endoderm derivatives, such as liver, can subsequently be detected (Fig. 2C), despite the presence of cardiac muscle (Fig. 2A).

WNT/β-catenin signalling opposes cardiogenesis by GATA4

Our finding of an apparent inverse relationship between endodermal and cardiac differentiation in explants has some parallels in studies of whole embryos. Endodermal development requires β-catenin (Lickert et al., 2002), whereas cardiac differentiation is inhibited by the β-catenin pathway (Lickert et al., 2002; Marvin et al., 2001; Schneider and Mercola, 2001). In agreement with this, we found that activation of the WNT pathway by XWNT8 during gastrulation completely blocks cardiac induction by GATA4 (Fig. 3A). The secreted antagonist of WNT signaling, DKK1 (Glinka et al., 1998), antagonises the WNT/β-catenin signalling pathway in animal pole explants, reducing transcription from a synthetic β-catenin/TCF-dependent reporter (Fig. 3E). When co-injected with *Gata4*, *Dkk1* has a synergistic effect on cardiac induction, whether *Gata4* was activated immediately upon explant isolation (late blastula stage) or delayed until the equivalent of early neurula stages (Fig. 3B). This suggests that the WNT/β-catenin pathway continuously antagonises GATA4 function during early development of injected explants. Interestingly, *Gata4* alone also led to a decrease in WNT/β-catenin pathway activity, and co-injection of *Gata4* and *Dkk1* resulted in an even greater reduction (Fig. 3E).

Extracellular BMP and Nodal factors are not required for GATA4-mediated cardiogenesis

Another extracellular antagonist of the WNT/β-catenin pathway is Cerberus (Bouwmeester et al., 1996). Co-

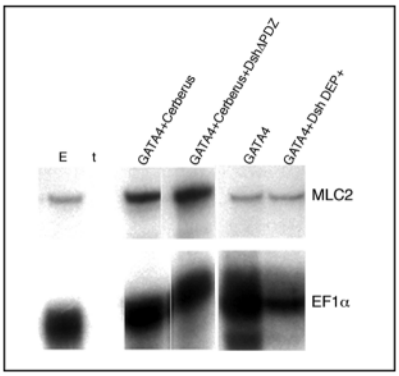

Fig. 4. The Dishevelled-dependent WNT/PCP pathway is not required for GATA4-mediated cardiogenesis. Injection of Dishevelled (Dsh) ΔPDZ dominant-negative construct, which inhibits both canonical and PCP WNT pathways, does not alter induction of the cardiac marker MLC2 by GATA4 and Cerberus. Similarly, Dishevelled DEP+, a PCP pathway-specific dominant-negative construct, does not affect induction of MLC2 by GATA4.

expression of this secreted protein with GATA4 in explants greatly stimulated cardiac induction (Fig. 3C) and the subsequent formation of beating tissue (see Movie 2 at http://dev.biologists.org/supplemental/). As in the case of DKK1, at least a part of the mechanism of action of Cerberus may involve a reduction of TCF-dependent transcription (Fig. 3E). However, in addition to binding and antagonising WNT, Cerebrus binds and antagonises the signalling factors BMP and Nodal (Piccolo et al., 1999). Our results therefore suggest that extracellular BMP and Nodal are not required for the cardiogenic activity of GATA4. Consistent with this, GATA4-induced cardiogenesis was unaffected by the presence of Chordin (Piccolo et al., 1996), another secreted antagonist of BMP (Fig. 3D).

GATA4-mediated cardiogenesis and the planar-cell polarity WNT pathway

Although signalling via the WNT/β-catenin pathway inhibits cardiac differentiation in embryos or explants, it has recently been proposed that non-canonical or planar-cell polarity (PCP) WNT signalling is required for cardiogenesis in vertebrates (Pandur et al., 2002). To test the role of this pathway in GATA4-mediated cardiac induction of explants, we used a dominant-negative mutant of Dishevelled, ΔPDZ, which has previously been shown to inhibit both canonical and non-canonical WNT pathways (Sokol, 1996; Tada and Smith, 2000). In order to obtain unequivocal results, we used co-injection of *Dkk1* or *Cerberus* with *Gata4* because this yields the most robust and reliable induction of cardiac markers (see above). Under such conditions, ΔPDZ had no effect on cardiac differentiation induced by GATA4 (Fig. 4), nor did the PCP pathway-specific dominant negative construct DEP+ (Tada and Smith, 2000). The Dishevelled-dependent non-canonical WNT pathway is not therefore required in the pathway by which GATA4 triggers cardiogenesis in animal pole explants.

NKX2.5 and GATA4-mediated cardiogenesis

The homeobox gene *Nkx2.5*, like its fly orthologue *tinman*, is believed to regulate early steps in cardiogenesis and is thought, in turn, to be regulated by BMP signalling (Harvey, 2002). In amphibian embryos, *Nkx2.5* expression is reportedly activated early during gastrulation and although the distribution of transcripts at this stage is poorly characterised, their accumulation is approximately coincident with the specification of cardiac precursors (Evans, 1999). We therefore investigated whether *Nkx2.5* is induced during GATA4-mediated cardiogenesis, and whether its expression is regulated by BMP signalling.

We found that *Nkx2.5* mRNA can frequently be detected in uninjected animal pole explants cultured until the equivalent of gastrula or neurula stage, although none was detected at later stages (Fig. 2B, Fig. 5 and data not shown). As a consequence, although no dramatic elevation of *Nkx2.5* levels was evident early in GATA4-expressing explants, we were unable to establish unambiguously whether GATA4 expression triggered a rapid but subtle elevation of *Nkx2.5* transcripts in explants. Elevated levels of *Nkx2.5* transcripts were detected at the equivalent of tadpole stages (Fig. 5B), but this may simply be

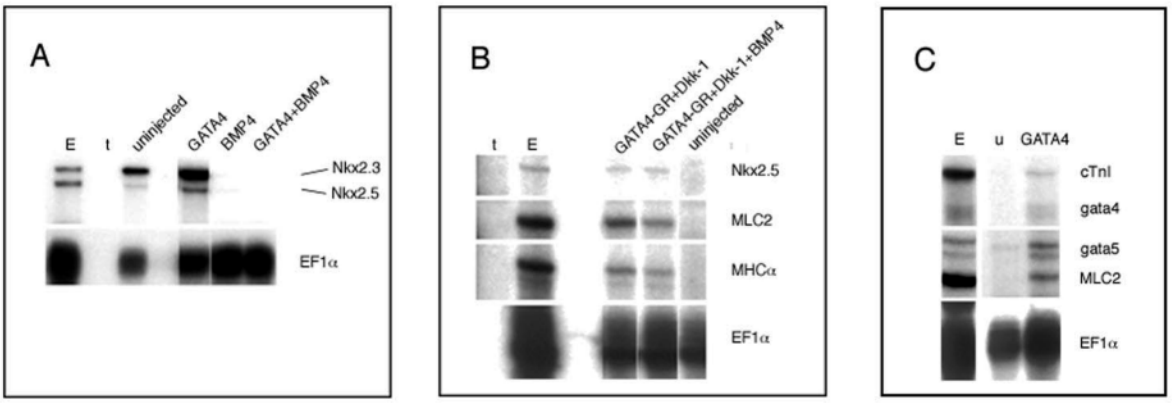

Fig. 5. BMP4 inhibits early *Nkx2.5* expression but not cardiogenesis: injected *Gata4* elevates endogenous *Gata4* and *Gata5* mRNA levels. (A) *Nkx2.5* and *Nkx2.3* are present in uninjected animal caps (stage 16). BMP4 leads to the disappearance of these transcripts in the presence of ectopic GATA4. (B) BMP4 does not prevent cardiogenesis induced by GATA4 in the presence of *Dkk1* or the elevation of *Nkx2.5* at tadpole stages (stage 37/38). (C) Injected *Gata4* induces endogenous *Gata4* and *Gata5* mRNA at the tadpole stage, along with cardiac markers. Both endogenous *Gata4* and *Gata5* mRNAs were detectable in uninjected explants at gastrula and neurula stages, at levels that were not significantly changed by ectopic GATA4 (data not shown).

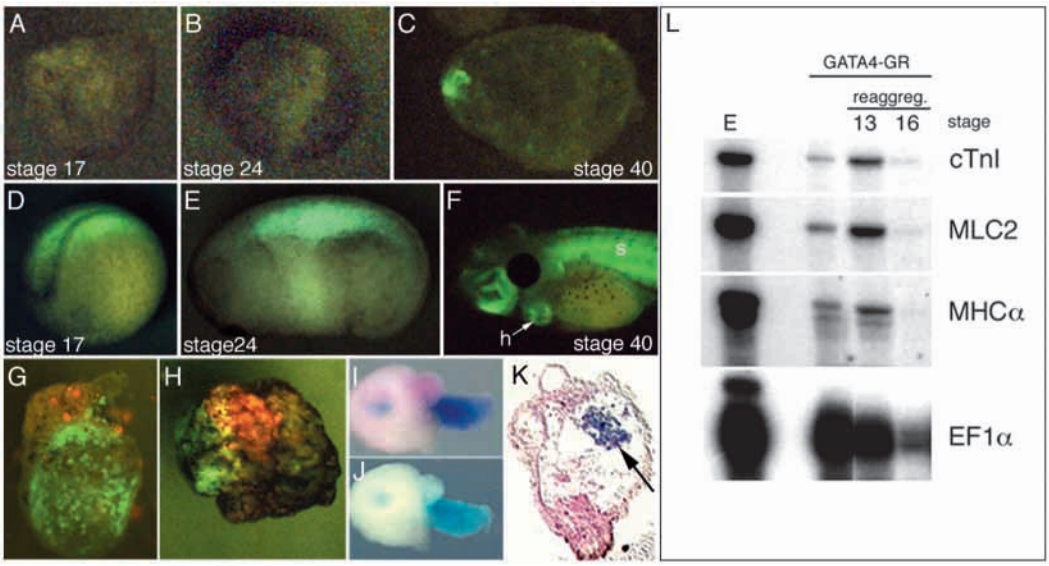

Fig. 6. GATA4 does not induce cardiac tissue precociously, and acts both non-cell- and cell-autonomously to induce cardiomyocytes. Cardiac actin-GFP transgenic embryos were injected with GATA4 (A-C), or with GATA4 together with rhodamine-dextran as a lineage tracer (G,H), in one blastomere at either the four- or eight-cell stage. Temporally correct cardiac differentiation was documented by observation of GFP. The cardiac actin promoter is active in both skeletal and cardiac muscle tissue. GATA4 does not induce skeletal muscle (Fig. 1), and it also fails to induce GFP at stages 17 (A) and 24 (B), when the transgene is active in skeletal muscle of sibling control embryos (D,E). GFP activity in GATA4-injected explants (C) is only detected when it is also present in the heart of sibling embryos (F). In G and H injected tissue was detected by rhodamine fluorescence (red); overlap with GFP is evident (yellow). Most GFP fluorescence is distinct from the rhodamine signal, which demonstrates the non-cell-autonomous action of GATA4. However, the areas of overlap also suggest cell-autonomous action of GATA4. (F) Stage 40 control sibling embryo showing cardiac-GFP expression in the heart (h) and somites (s). (I-K) Embryos were injected with GATA4 together with Cerberus in one blastomere at the four- or eight-cell stage, and biotinylated dextran was co-injected as a lineage tracer. MLC2 expression at stage 38 was detected by in situ hybridisation (pale blue in J), and the injected part of explant is revealed in whole mount (I) or on sections (K) as magenta staining. Overlap of the two colours creates a purple signal (arrow), which indicates a cell-autonomous action of GATA4. Embryos are shown with the anterior end to the left. (L) Cell-cell interactions are not required for GATA4 action until at least stage 16. Animal pole explants injected with GATA4-GR were dispersed in Ca^{2+}/Mg^{2+}-free medium after excision. GATA4 was subsequently activated by dexamethasone. Single-cell suspensions were cultured with constant gentle agitation to prevent spontaneous formation of cell contacts. Cells were re-aggregated at an indicated stage by the addition of Ca^{2+} and pelleting, and were cultured until sibling controls reached stage 37/38. Note that viability of cells kept in suspension until stage 16 is low, as revealed by poor EF1α recovery; nevertheless, cardiac markers were still detectable.

a consequence of cardiac tissue differentiation rather than an indication that the effects of GATA4 are mediated via *Nkx2.5* expression.

Co-injection of *Gata4* and *Bmp4* mRNA in animal caps led to a decline rather than an increase in the level of *Nkx2.5* mRNA in early explants (Fig. 5A). Interestingly, despite the absence of *Nkx2.5* in these explants during early stages, elevated levels were later detected along with cardiac muscle markers, and subsequent cardiogenesis was unaffected (Fig. 5B). These results indicate that detectable levels of *Nkx2.5* transcripts are not required at early stages in the development of GATA4-expressing explants for cardiac differentiation to be triggered, nor is the cardiogenic activity of GATA4 affected by elevated BMP4 expression.

GATA4 acts both cell-autonomously and non-cell-autonomously

Is GATA4 acting only in explant cells that express it, or could it be inducing adjacent non-expressing cells to adopt a cardiac fate? We examined this question by creating a localised signalling source in explants. This was achieved by injecting *Gata4*, together with a permanent lineage tracer, into a single blastomere of the four- or eight-cell embryo. Explants subsequently removed from such embryos were mosaic for GATA4 expression, as revealed by the distribution of lineage marker. Under these conditions, cardiac tissue was generated by GATA4 in both lineage labelled and unlabelled tissue (Fig. 6), indicating that GATA4-expressing cells had signalled to adjacent ectodermal cells to adopt a cardiomyocyte fate. Some GATA4-expressing cells themselves became cardiac tissue and this cell-autonomous activity of GATA4 was predominant when GATA4 was co-expressed with Cerberus (Fig. 6I-K).

We also tested directly the importance of cell-cell interactions for GATA4-meditaed cardiogenesis, by dispersing explants into single-cell suspensions. This experimental approach is limited because prolonged incubation of single-cell suspensions leads to increasing loss of cell viability. Nevertheless, we found that cell-cell interactions are not required at least until stage 16 (Fig. 6L), after which re-aggregation of cells was necessary for continued culture to be successful.

DISCUSSION

In this report we show that cardiomyocytes can be induced in explants of presumptive ectodermal tissue from *Xenopus*

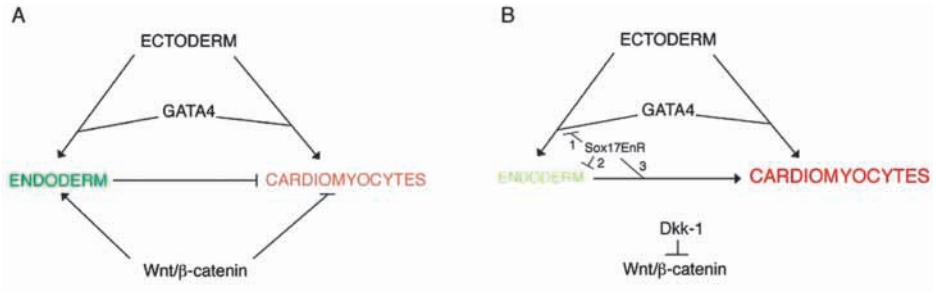

Fig. 7. A model of the action of GATA4 in ectodermal explants. (A) GATA4 converts embryonic ectoderm to both endodermal and cardiomyocyte fates. Endoderm and WNT/β-catenin antagonise cardiac tissue formation. (B) The dominant-negative *Sox17* construct and the WNT antagonist *Dkk1* each promote cardiomyocyte fate at the expense of endodermal fate. *Sox17βEnR* could inhibit the endoderm-inducing activity and promote the cardiogenic activity of GATA4 directly (1), and/or prevent the maintenance of endoderm (2), and/or convert endoderm to cardiomyocytes (3). WNT/β-catenin could interfere with cardiogenesis either directly or indirectly by promoting endoderm formation (Lickert et al., 2002).

embryos by ectopic expression of GATA4. Expression of cardiomyocyte markers is neither precocious nor selective, indicating that their expression reflects genuine induction of cardiac muscle tissue rather than the selective transactivation of myocardial genes containing GATA-factor binding sites. GATA4 apparently triggers the formation of several mesendodermal tissues, including cardiac, but not skeletal, muscle.

GATA factors and cardiogenesis

Several lines of evidence have already indicated the importance of GATA factors in heart development. Potential GATA binding sites are commonly found in cardiac gene regulatory regions, and, in transfection assays, GATA4, GATA5 and GATA6 have each been found to activate a variety of cardiac-specific promoters (Charron and Nemer, 1999; Molkentin, 2000; Patient and McGhee, 2002). Genetic analyses of the role of GATA4, GATA5 and GATA6 in heart development have been complicated by their requirement in other tissues, as well as by the potential for redundancy between them, which results from overlapping expression patterns and similar activities. In the mouse, homozygous-mutant *Gata4* embryos developed cardia bifida (Kuo et al., 1997; Molkentin et al., 1997), *Gata5* mutant mice develop normally (Molkentin et al., 2000) and *Gata6* mutation results in lethality at the time of implantation (Koutsourakis et al., 1999; Morrisey et al., 1998). The phenotypes of compound and tissue-restricted mutants have not been reported yet. In zebrafish, *Gata5* (*faust*) mutants show a combination of cardiac bifida and a severe reduction in the number of ventricular myocytes that form (Reiter et al., 1999). In gain-of-function studies in P19 embryonic carcinoma cells, GATA4 promotes cardiogenesis (Grepin et al., 1997). P19 cells have a propensity to differentiate into several lineages, including cardiomyocytes, and this complicates interpretation of the mode of GATA4 action.

In addition to a role in cardiac differentiation, there is also evidence that GATA factors are important regulators of endoderm differentiation. GATA4, GATA5 and GATA6 can activate some endodermal promoters in vitro (Gao et al., 1998; Patient and McGhee, 2002) and the cardiac bifida of GATA4 null mice can be rescued by wild-type endodermal cells in chimaeras (Narita et al., 1997). The cardiac phenotype resulting from *Gata5* mutation in *faust* zebrafish (which combines cardiac bifida with a loss of myocardial tissue) is likely to be a composite, resulting from the lack of appropriate GATA function in both cardiac and endodermal precursors (Reiter et al., 1999).

A role of GATA4, GATA5 and GATA6 factors in endoderm formation is further supported by findings that they trigger endoderm differentiation in vitro. In *Xenopus* ectodermal explants, GATA5 induces both early and late endodermal markers (Weber et al., 2000). Our results show that GATA4 and, most strikingly, GATA6 have the same effect. In ES cells, GATA4 and GATA6 promote differentiation of extra-embryonic endoderm (Fujikura et al., 2002). This can occur in the presence of LIF (which blocks spontaneous differentiation of these cells) and does not require the complex cellular environment of embryoid bodies. We have shown that in addition to inducing endoderm, GATA4 and GATA5 induce cardiac tissue, and together these findings lend support to a model in which both mesodermal and endodermal tissues in the early embryo arise from bipotential mesendodermal progenitors (Lickert et al., 2002; Rodaway and Patient, 2001). Interestingly, we find that GATA4-expressing explants show elevated levels of endogenous *Gata4* and *Gata5* transcripts (Fig. 5C). Similarly, activation of endogenous *Gata4* and *Gata6* occurs during GATA4-mediated endodermal conversion of ES cells (Fujikura et al., 2002). In each case, the activity of ectopic GATA4 may therefore be reinforced by a positive auto-regulatory loop.

Is the induction of cardiac tissue in GATA4-expressing animal pole explants a secondary consequence of mesendodermal tissue differentiation or does this result indicate a more direct link between GATA function and specification of cardiac fate? In zebrafish embryos, overexpression of Gata5 results in the formation of ectopic cardiac muscle tissue, and this can occur in a cell-autonomous manner (Reiter et al., 1999) consistent with direct effect of the factor. Although we find no evidence for a similar effect of GATA4, GATA5 and GATA6 in frog embryos (Gove et al., 1997) (data not shown), several of our findings nevertheless lend support to the view that the cardiogenic action of GATA factors in explants is relatively direct (as discussed below).

Endoderm and cardiac induction in explants

The first of these is the finding that induction of cardiac tissue can occur in the apparent absence of endoderm. Ectopic expression of GATA4 in animal pole explants results in the formation endodermal tissues such as gut (Weber et al., 2000) and liver (Figs 2, 3). Because signalling from endodermal tissue appears to underlie specification of cardiac mesoderm (Lough and Sugi, 2000; Nascone and Mercola, 1995), we might expect that the presence of endoderm in explants would

be essential for GATA4-mediated induction of cardiac tissue. However, we have found the opposite to be true; inhibition of endoderm differentiation by a dominant-negative form of the endoderm transcription factor SOX17β actually increases, rather than decreases, the amount of cardiac tissue that is formed. This suggests that GATA4-mediated induction of cardiac progenitors in explants may occur in the absence of an endodermal tissue. Whether cardiac specification under such circumstances occurs without the molecular cues normally provided by endodermal cells (i.e. GATA4 acts downstream of endodermal signal), or whether GATA4 can also mediate these signals independently, is unresolved by our experiments.

Our finding that cardiac differentiation is substantially enhanced as a result of *Sox17βEnR* expression suggests that the formation of SOX17β-dependent endoderm and cardiac progenitors are mutually antagonistic in GATA4-expressing explants. Alternatively, or in addition, SOX17βEnR may lead to a shift in cell fate from endoderm to mesoderm. Such a shift in fate caused by SOX17βEnR in *Xenopu*s embryos has been reported (Clements and Woodland, 2000). A simple model shown in Fig. 7 is that cardiac mesoderm and endoderm are alternative developmental pathways for common precursors. *Sox17βEnR* may enhance cardiogenesis directly by opposing the endoderm-inducing activity of GATA4, by blocking maintenance of endoderm, and/or by causing a shift in fate (Fig. 7).

SOX17β and the related SOX17α are thought to be pan-endodermal, and for this reason *Sox17βEnR* may be expected to inhibit all endoderm in *Xenopus* (Hudson et al., 1997). However, recent genetic analyses in the mouse have shown that anterior endoderm is *Sox17*-independent in that organism (Kanai-Azuma et al., 2002). We cannot therefore exclude the possibility that, in *Xenopus* explants expressing GATA4 and SOX17βEnR, sufficient endoderm formation still occurs to induce cardiac progenitors. If so, such tissue is likely to be transient because we were unable to detect liver or pancreas endoderm in explants expressing GATA4 and SOX17βEnR.

Cell-autonomous activity of GATA4

A second reason for considering that the induction of cardiac tissue by GATA4 might not simply be an indirect consequence of mesendoderm formation comes from our lineage labelling results. The ability of GATA4 to induce cardiac tissue in cells that have not received the injected GATA4 transcript indicates that specification of cardiac fate within explants can occur through cell-to-cell interactions (as might indeed be expected if endodermal signalling was required). Of course, similar cell-to-cell interactions could also result in cell-autonomous induction of cardiac tissue if both signalling and responding cells were derived from GATA4-expressing explant tissue. However, cell-autonomous induction may also indicate a more direct mechanism by which GATA4 triggers cardiac specification. Such an interpretation is consistent with earlier findings that GATA5 can act both cell-autonomously and non-cell-autonomously to induce ectopic myocardium in zebrafish embryos (Reiter et al., 1999). It is also supported by our observation that in explants co-expressing Cerberus and GATA4 virtually all cardiac tissue is derived cell-autonomously (Fig. 6). Cerberus not only enhances the extent of cardiac differentiation in GATA4-expressing explants but also inhibits the formation of endoderm. It should therefore suppress cardiac induction that is mediated by endodermal signalling.

The timing of GATA4-mediated cardiac induction

Our investigation of when GATA4-mediated induction can occur using a dexamethasone-inducible form of the transcription factor has yielded intriguing results. Whereas normal cardiogenic signals are restricted to a brief period during gastrulation, in explants we find that cardiac differentiation can be triggered by GATA4 even after the onset of terminal epidermal differentiation. Under such circumstances, GATA4 might be converting epidermal cells towards cardiomyocyte fate, or it could be acting on a hypothetical population of stem-like cells. In either case, the finding is remarkable, and it is clearly important to distinguish between these possibilities in future studies. Equally important is to establish whether such late-onset cardiac induction occurs cell-autonomously and whether it is necessarily accompanied by endoderm differentiation. As yet we have not resolved the former issue but find little SOX17α expression and no liver marker LFABP after delayed activation of the inducible *Gata4* (Fig. 2).

Signalling pathways for cardiac specification

WNT signalling

Studies using explants of non-cardiac mesoderm from chick and frog embryos have indicated a role for antagonists of WNT/β-catenin signalling (DKK1 and Crescent) in the formation of cardiac progenitor tissue (Marvin et al., 2001; Schneider and Mercola, 2001). Our finding that DKK1 has a similar stimulatory effect on cardiac induction indicates that inhibition of cardiogenesis by the WNT/β-catenin pathway occurs at the level of GATA4 or downstream of it. Inhibition may occur directly, by targeting cardiac progenitors, or indirectly via its effect on endoderm formation (Fig. 7). An indirect mode of action is supported by the study of Lickert et al., who have shown that the inhibition of WNT/β-catenin signalling in mouse embryos leads to formation of ectopic cardiac tissue and loss of posterior endoderm (Lickert et al., 2002) (see Fig. 7). Even when GATA4 activation in explants is delayed until control embryos form neurulae, *Dkk1* expression still enhances GATA4-mediated cardiogenesis, This result indicates that GATA4 operates during both gastrula and neurula stages in the presence of inhibitory WNT/β-catenin signalling.

In addition to WNT/β-catenin signalling, the non-canonical or planar-cell polarity WNT pathway has also been implicated in regulating cardiogenesis (Pandur et al., 2002). Reduction of WNT11 protein levels in *Xenopus* embryos leads to defects in heart formation, and ectopic expression of *Wnt11* mRNA in posterior mesoderm (ventral marginal zone) explants results in formation of ectopic beating tissue. A similar effect was reported with animal pole explants, but only in the presence of the mesendodermal inducer activin. In our experiments, we find that inhibition of the WNT/PCP pathway at the level of Dishevelled has no effect on cardiomyocyte induction by GATA4. The reason for such a discrepancy between our results and those previously reported is unclear. One possible explanation is that the Dishevelled-dependent WNT/PCP pathway acts upstream of GATA4 in cardiogenesis.

BMP pathway

The earliest indicator of formation of cardiac progenitors is the expression of the *tinman* homologue *Nkx2.5* and it has been proposed that this is the result of BMP signalling (Harvey, 2002). In our assay, we have found that the secreted antagonists of BMP signalling, Cerberus and Chordin, do not, in fact, block GATA4-mediated cardiogenesis (Fig. 3). Neither does expression of a dominant-negative BMP receptor (B.V.L. and T.J.M., unpublished). This could be interpreted to indicate that GATA4-mediated cardiac induction in explants occurs in a fundamentally different manner to cardiogenesis in embryos. Alternatively, it could indicate that BMP signalling lies upstream of GATA4 in the cardiac induction pathway. Consistent with this interpretation, Gata5 has been shown to act downstream of Bmp2 in regulation of Nkx2.5 in the zebrafish embryo (Reiter et al., 2001).

Our efforts to establish the relationship between GATA4-mediated induction and *Nkx2.5* expression were hampered by the detection of endogenous transcripts in control animal pole explants. However, no obvious elevation of *Nkx2.5* transcripts could be detected as an early response to GATA4 expression, a result that is all the more striking because GATA factors are thought to be key regulators of *Nkx2.5* transcription in vivo (Reecy et al., 1999; Searcy et al., 1998; Sparrow et al., 2000). Whether GATA4-mediated cardiac induction bypasses *Nkx2.5* or whether subtle changes in *Nkx2.5* expression are sufficient for triggering subsequent cardiac differentiation remains to be determined, but it is noteworthy that cardiac induction still occurs in explants expressing BMP4 and GATA4 despite a suppression of *Nkx2.5* transcript levels (Fig. 5).

Conclusion

Expression of GATA4 in *Xenopus* blastula animal pole explants provides a simple and reliable means of inducing cardiomyocytes. We do not yet know whether this reflects a role for GATA4 in normal embryogenesis or whether ectopic expression of this factor mimics or bypasses endogenous inducing signals. In either case, such explants offer a convenient model system to study the molecular signals regulating myocardial differentiation. Mammalian stem cells differentiate to cardiac fate at only a low frequency, either spontaneously or in response to treatments that are neither physiological nor easy to control (Daley, 2002; Schuldiner et al., 2000). Our assay might therefore help to define rational strategies for efficient direction of pluripotent cells to a cardiomyocyte fate.

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REFERENCES

Alexander, J., Rothenberg, M., Henry, G. L. and Stainier, D. Y. (1999). casanova plays an early and essential role in endoderm formation in zebrafish. *Dev. Biol.* **215**, 343-357.

Benson, D. W., Silberbach, G. M., Kavanaugh-McHugh, A., Cottrill, C., Zhang, Y., Riggs, S., Smalls, O., Johnson, M. C., Watson, M. S., Seidman, J. G. et al. (1999). Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. *J. Clin. Invest.* **104**, 1567-1573.

Biben, C. and Harvey, R. P. (1997). Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene eHand during murine heart development. *Genes Dev.* **11**, 1357-1369.

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Breckenridge, R. A., Mohun, T. J. and Amaya, E. (2001). A role for BMP signalling in heart looping morphogenesis in Xenopus. *Dev. Biol.* **232**, 191-203.

Bruneau, B. G. (2002). Transcriptional regulation of vertebrate cardiac morphogenesis. *Circ. Res.* **90**, 509-519.

Chambers, A. E., Logan, M., Kotecha, S., Towers, N., Sparrow, D. and Mohun, T. J. (1994). The RSRF/MEF2 protein SL1 regulates cardiac muscle-specific transcription of a myosin light-chain gene in Xenopus embryos. *Genes Dev.* **8**, 1324-1334.

Charron, F. and Nemer, M. (1999). GATA transcription factors and cardiac development. *Semin. Cell Dev. Biol.* **10**, 85-91.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Clements, D. and Woodland, H. R. (2000). Changes in embryonic cell fate produced by expression of an endodermal transcription factor, Xsox17. *Mech. Dev.* **99**, 65-70.

Cripps, R. M. and Olson, E. N. (2002). Control of cardiac development by an evolutionarily conserved transcriptional network. *Dev. Biol.* **246**, 14-28.

Dale, L., Howes, G., Price, B. M. and Smith, J. C. (1992). Bone morphogenetic protein 4: a ventralizing factor in early *Xenopus* development. *Development* **115**, 573-585.

Dale, L. and Jones, C. M. (1999). BMP signalling in early Xenopus development. *BioEssays* **21**, 751-760.

Daley, G. Q. (2002). Prospects for stem cell therapeutics: myths and medicines. *Curr. Opin. Genet. Dev.* **12**, 607-613.

Drysdale, T. A., Tonissen, K. F., Patterson, K. D., Crawford, M. J. and Krieg, P. A. (1994). Cardiac troponin I is a heart-specific marker in the Xenopus embryo: expression during abnormal heart morphogenesis. *Dev. Biol.* **165**, 432-441.

Evans, S. M. (1999). Vertebrate tinman homologues and cardiac differentiation. *Semin. Cell Dev. Biol.* **10**, 73-83.

Evans, S. M., Yan, W., Murillo, M. P., Ponce, J. and Papalopulu, N. (1995). tinman, a *Drosophila* homeobox gene required for heart and visceral mesoderm specification, may be represented by a family of genes in vertebrates: XNkx-2.3, a second vertebrate homologue of tinman. *Development* **121**, 3889-3899.

Fujikura, J., Yamato, E., Yonemura, S., Hosoda, K., Masui, S., Nakao, K., Miyazaki Ji, J. and Niwa, H. (2002). Differentiation of embryonic stem cells is induced by GATA factors. *Genes Dev.* **16**, 784-789.

Gao, X., Sedgwick, T., Shi, Y. B. and Evans, T. (1998). Distinct functions are implicated for the GATA-4, -5, and -6 transcription factors in the regulation of intestine epithelial cell differentiation. *Mol. Cell. Biol.* **18**, 2901-2911.

Gillespie, L. L., Paterno, G. D. and Slack, J. M. (1989). Analysis of competence: receptors for fibroblast growth factor in early *Xenopus* embryos. *Development* **106**, 203-208.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.

Gove, C., Walmsley, M., Nijjar, S., Bertwistle, D., Guille, M., Partington, G., Bomford, A. and Patient, R. (1997). Over-expression of GATA-6 in Xenopus embryos blocks differentiation of heart precursors. *EMBO J.* **16**, 355-368.

Green, J. B., New, H. V. and Smith, J. C. (1992). Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* **71**, 731-739.

Grepin, C., Nemer, G. and Nemer, M. (1997). Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* **124**, 2387-2395.

Grimm, O. H. and Gurdon, J. B. (2002). Nuclear exclusion of Smad2 is a mechanism leading to loss of competence. *Nat. Cell Biol.* **4**, 519-522.

Gurdon, J. B., Mitchell, A. and Ryan, K. (1996). An experimental system

for analyzing response to a morphogen gradient. *Proc. Natl. Acad. Sci. USA* **93**, 9334-9338.
Harvey, R. P. (2002). Patterning the vertebrate heart. *Nat. Rev. Genet.* **3**, 544-556.
Henry, G. L. and Melton, D. A. (1998). Mixer, a homeobox gene required for endoderm development. *Science* **281**, 91-96.
Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). MyoD expression in the forming somites is an early response to mesoderm induction in Xenopus embryos. *EMBO J.* **8**, 3409-3417.
Horb, M. E. and Slack, J. M. (2002). Expression of amylase and other pancreatic genes in Xenopus. *Mech. Dev.* **113**, 153-157.
Huber, T. L. and Zon, L. I. (1998). Transcriptional regulation of blood formation during Xenopus development. *Semin. Immunol.* **10**, 103-109.
Hudson, C., Clements, D., Friday, R. V., Stott, D. and Woodland, H. R. (1997). Xsox17alpha and -beta mediate endoderm formation in Xenopus. *Cell* **91**, 397-405.
Jiang, Y. and Evans, T. (1996). The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. *Dev. Biol.* **174**, 258-270.
Jonas, E., Sargent, T. D. and Dawid, I. B. (1985). Epidermal keratin gene expressed in embryos of Xenopus laevis. *Proc. Natl. Acad. Sci. USA* **82**, 5413-5417.
Kanai-Azuma, M., Kanai, Y., Gad, J. M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P. P. et al. (2002). Depletion of definitive gut endoderm in Sox17-null mutant mice. *Development* **129**, 2367-2379.
Kikuchi, Y., Agathon, A., Alexander, J., Thisse, C., Waldron, S., Yelon, D., Thisse, B. and Stainier, D. Y. (2001). casanova encodes a novel Sox-related protein necessary and sufficient for early endoderm formation in zebrafish. *Genes Dev.* **15**, 1493-1505.
Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R. and Grosveld, F. (1999). The transcription factor GATA6 is essential for early extraembryonic development. *Development* **126**, 723-732.
Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048-1060.
Latinkić, B. V., Cooper, B., Towers, N., Sparrow, D., Kotecha, S. and Muhun, T. J. (2002). Distinct enhancers regulate skeletal and cardiac muscle-specific expression programs of the cardiac α-actin gene in *Xenopus* embryos. *Dev. Biol.* **245**, 57-70.
Lickert, H., Kutsch, S., Kanzler, B., Tamai, Y., Taketo, M. M. and Kemler, R. (2002). Formation of multiple hearts in mice following deletion of beta-catenin in the embryonic endoderm. *Dev. Cell* **3**, 171-181.
Logan, M. and Mohun, T. (1993). Induction of cardiac muscle differentiation in isolated animal pole explants of *Xenopus laevis* embryos. *Development* **118**, 865-875.
Lough, J. and Sugi, Y. (2000). Endoderm and heart development. *Dev. Dyn.* **217**, 327-342.
Marvin, M. J., Di Rocco, G., Gardiner, A., Bush, S. M. and Lassar, A. B. (2001). Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Genes Dev.* **15**, 316-327.
Mead, P. E., Deconinck, A. E., Huber, T. L., Orkin, S. H. and Zon, L. I. (2001). Primitive erythropoiesis in the *Xenopus* embryo: the synergistic role of LMO-2, SCL and GATA-binding proteins. *Development* **128**, 2301-2308.
Mironov, V. N., Van Montagu, M. and Inze, D. (1995). High throughput RNase protection assay. *Nucl. Acids Res.* **23**, 3359-3360.
Molkentin, J. D. (2000). The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *J. Biol. Chem.* **275**, 38949-38952.
Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11**, 1061-1072.
Molkentin, J. D., Tymitz, K. M., Richardson, J. A. and Olson, E. N. (2000). Abnormalities of the genitourinary tract in female mice lacking GATA5. *Mol. Cell. Biol.* **20**, 5256-5260.
Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S. and Parmacek, M. S. (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12**, 3579-3590.
Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C. P. et al. (1996). Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. *Development* **123**, 81-93.
Narita, N., Bielinska, M. and Wilson, D. B. (1997). Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. *Dev. Biol.* **189**, 270-274.
Nascone, N. and Mercola, M. (1995). An inductive role for the endoderm in *Xenopus* cardiogenesis. *Development* **121**, 515-523.
Pandur, P., Lasche, M., Eisenberg, L. M. and Kuhl, M. (2002). Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* **418**, 636-641.
Patient, R. K., Banville, D., Brewer, A. C., Elkington, J. A., Greaves, D. R., Lloyd, M. M. and Williams, J. G. (1982). The organization of the tadpole and adult alpha globin genes of Xenopus laevis. *Nucl. Acids Res.* **10**, 7935-7945.
Patient, R. K. and McGhee, J. D. (2002). The GATA family (vertebrates and invertebrates). *Curr. Opin. Genet. Dev.* **12**, 416-422.
Piccolo, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-598.
Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-710.
Reecy, J. M., Li, X., Yamada, M., DeMayo, F. J., Newman, C. S., Harvey, R. P. and Schwartz, R. J. (1999). Identification of upstream regulatory regions in the heart-expressed homeobox gene Nkx2-5. *Development* **126**, 839-849.
Reiter, J. F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N. and Stainier, D. Y. (1999). Gata5 is required for the development of the heart and endoderm in zebrafish. *Genes Dev.* **13**, 2983-2995.
Reiter, J. F., Verkade, H. and Stainier, D. Y. (2001). Bmp2b and Oep promote early myocardial differentiation through their regulation of gata5. *Dev. Biol.* **234**, 330-338.
Rodaway, A. and Patient, R. (2001). Mesendoderm. An ancient germ layer? *Cell* **105**, 169-172.
Ryan, K., Garrett, N., Mitchell, A. and Gurdon, J. B. (1996). Eomesodermin, a key early gene in Xenopus mesoderm differentiation. *Cell* **87**, 989-1000.
Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.
Schlange, T., Andree, B., Arnold, H. H. and Brand, T. (2000). BMP2 is required for early heart development during a distinct time period. *Mech. Dev.* **91**, 259-270.
Schneider, M. D., Gaussin, V. and Lyons, K. M. (2003). Tempting fate: BMP signals for cardiac morphogenesis. *Cytokine Growth Factor Rev.* **14**, 1-4.
Schneider, V. A. and Mercola, M. (2001). Wnt antagonism initiates cardiogenesis in Xenopus laevis. *Genes Dev.* **15**, 304-315.
Schott, J. J., Benson, D. W., Basson, C. T., Pease, W., Silberbach, G. M., Moak, J. P., Maron, B. J., Seidman, C. E. and Seidman, J. G. (1998). Congenital heart disease caused by mutations in the transcription factor NKX2-5. *Science* **281**, 108-111.
Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D. A. and Benvenisty, N. (2000). Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **97**, 11307-11312.
Schultheiss, T. M., Burch, J. B. and Lassar, A. B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev.* **11**, 451-462.
Searcy, R. D., Vincent, E. B., Liberatore, C. M. and Yutzey, K. E. (1998). A GATA-dependent nkx-2.5 regulatory element activates early cardiac gene expression in transgenic mice. *Development* **125**, 4461-4470.
Seo, Y. W., Sanyal, S., Kim, H. J., Won, D. H., An, J. Y., Amano, T., Zavacki, A. M., Kwon, H. B., Shi, Y. B., Kim, W. S. et al. (2002). FOR, a novel orphan nuclear receptor related to farnesoid X receptor. *J. Biol. Chem.* **277**, 17836-17844.
Shi, Y., Katsev, S., Cai, C. and Evans, S. (2000). BMP signaling is required for heart formation in vertebrates. *Dev. Biol.* **224**, 226-237.
Shi, Y. B. and Hayes, W. P. (1994). Thyroid hormone-dependent regulation of the intestinal fatty acid-binding protein gene during amphibian metamorphosis. *Dev. Biol.* **161**, 48-58.
Sive, H. L., Grainger, R. M. and Harland, R. M. (2000). *Early Development of* Xenopus laevis*: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Smith, W. C. and Harland, R. M. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753-765.

Sokol, S. Y. (1996). Analysis of Dishevelled signalling pathways during Xenopus development. *Curr. Biol.* **6**, 1456-1467.

Sparrow, D. B., Cai, C., Kotecha, S., Latinkić, B., Cooper, B., Towers, N., Evans, S. M. and Mohun, T. J. (2000). Regulation of the tinman homologues in Xenopus embryos. *Dev. Biol.* **227**, 65-79.

Tada, M. and Smith, J. C. (2000). Xwnt11 is a target of *Xenopus* Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* **127**, 2227-2238.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N. and Izumo, S. (1999). The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* **126**, 1269-1280.

Theze, N., Hardy, S., Wilson, R., Allo, M. R., Mohun, T. and Thiebaud, P. (1995). The MLC1f/3f gene is an early marker of somitic muscle differentiation in Xenopus laevis embryo. *Dev. Biol.* **171**, 352-362.

Tonissen, K. F., Drysdale, T. A., Lints, T. J., Harvey, R. P. and Krieg, P. A. (1994). XNkx-2.5, a Xenopus gene related to Nkx-2.5 and tinman: evidence for a conserved role in cardiac development. *Dev. Biol.* **162**, 325-328.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A. et al. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **88**, 789-799.

Walters, M. J., Wayman, G. A. and Christian, J. L. (2001). Bone morphogenetic protein function is required for terminal differentiation of the heart but not for early expression of cardiac marker genes. *Mech. Dev.* **100**, 263-273.

Weber, H., Symes, C. E., Walmsley, M. E., Rodaway, A. R. and Patient, R. K. (2000). A role for GATA5 in *Xenopus* endoderm specification. *Development* **127**, 4345-4360.

Zhang, C. and Evans, T. (1994). Differential regulation of the two xGATA-1 genes during Xenopus development. *J. Biol. Chem.* **269**, 478-484.