Published in final edited form as:
Dev Biol. 2008 May 15; 317(2): 614–619. doi:10.1016/j.ydbio.2008.03.013.

Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice

Roong Zhao[1], **Alistair J. Watt**[1,2], **Michele A. Battle**[1], **Jixuan Li**[1], **Benjamin J. Bondow**[1], and **Stephen A. Duncan.**[1]

1 *Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin, USA*

2 *John Hughes Bennett Laboratory, University of Edinburgh, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh, UK*

Abstract

Despite significant advances in identifying signaling molecules that induce cardiogenesis in mammals, the transcription factors that control the onset of cardiac myocyte gene expression have remained elusive. Candidates include the zinc finger transcription factors GATA binding proteins 4 and 6 (GATA4, GATA6). The individual loss of either protein in mice results in lethality prior to the onset of heart development due to defects in the extra-embryonic endoderm; however, when this extra-embryonic deficiency is circumvented using tetraploid embryo complementation, cardiac myocyte differentiation initiates normally. Here we show that these factors have redundant roles in controlling the onset of cardiac myocyte differentiation. As a consequence, *Gata4*$^{-/-}$*Gata6*$^{-/-}$ embryos completely lack hearts, although second heart field progenitor cells are still generated. Our data support a model whereby GATA4 or GATA6 are essential for expression of the network of transcription factors that regulate the onset of cardiac myocyte gene expression during mammalian development.

Keywords

tetraploid; heart development; transcription factors; GATA4; GATA6

INTRODUCTION

Studies in *Drosophila melanogaster*, *Danio rerio* and *Xenopus laevis* have implicated several families of transcription factors in initiating cardiac myocyte gene expression in response to inductive signals, including the Nkx, Tbx, Mef, and GATA families as well as SRF (Olson and Schneider, 2003). However, no single mutation has been identified that blocks cardiac myocyte differentiation in mammals, suggesting that functional redundancy between factors exists. The GATA binding proteins are zinc finger transcription factors that have been implicated in regulating the onset of cardiac myocyte differentiation (Pikkarainen et al., 2004). GATA4, 5 and 6 are all expressed in cardiac progenitor cells, and all three proteins, although individually

Corresponding author: Stephen A. Duncan, D.Phil, Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI, 53226, Phone: 414 456 8602, Fax: 414 456 6517, e-mail: duncans@mcw.edu.

dispensable for cardiac myocyte formation, have critical roles in regulating heart development in zebrafish and frogs (Holtzinger and Evans, 2005; Peterkin et al., 2003; Reiter et al., 1999). More recently, morpholino-mediated depletion of combinations of GATA factors has resulted in preventing aspects of cardiac specification in Zebrafish and Xenopus suggesting that the GATA factors have redundant but essential roles in controlling early stages of heart development in these species (Holtzinger and Evans, 2007; Peterkin et al., 2007). Elucidating the contribution the GATA factors make to the development of the mammalian heart has been challenging. Gene knockout studies in mice demonstrated that GATA5 was dispensable for embryonic development (Molkentin et al., 2000), while loss of either GATA4 or GATA6 resulted in a developmental arrest during gastrulation due to a requirement for these factors in the extra-embryonic endoderm (Koutsourakis et al., 1999; Kuo et al., 1997; Molkentin et al., 1997; Morrisey et al., 1998). When this early embryonic lethality was circumvented by providing GATA4 or GATA6 null embryos with wild type extra-embryonic endoderm, formation of the early heart and cardiac myocyte gene expression appeared relatively normal, although subtle deficiencies in maturation of the myocytes were identified (Narita et al., 1997b; Watt et al., 2004; Zhao et al., 2005). GATA4 and GATA6 are highly conserved, are capable of binding identical nucleotide sequences in genomic DNA, and regulate expression of similar target genes (Morrisey et al., 1997; Nemer and Nemer, 2003; Pikkarainen et al., 2004). We therefore sought to test the proposal that these factors had redundant functions in controlling mammalian cardiac myocyte differentiation and predicted that if both factors were simultaneously disrupted, the onset of cardiac development would be blocked.

MATERIALS AND METHODS

Generation of *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ ES cell lines, embryoid bodies and embryos

The production of *Gata4*$^{-/-}$ and *Gata6*$^{-/-}$ ES cells have been described previously (Watt et al., 2004; Zhao et al., 2005). To generate *Gata4*$^{-/-}$*Gata6*$^{-/-}$ ES cells, we first produced a targeting vector, pGATA4loxPDT, that contains a *Neo-tk* cassette flanked by two *lox*P sites (sites a and b) from plasmid pHR-1 that was inserted into the *SmaI* site 85bp upstream of *Gata4* exon 3 (Watt et al., 2004). In addition, a single *lox*P site (site c) from plasmid pHR-1 was inserted into the *BamHI* site between exons 5 & 6. Negative selection was provided by the diptheria toxin gene. Cre mediated recombination between the two outermost *lox*P sites deletes both zinc finger domains and the transactivation domain of GATA4 resulting in complete loss of function and the absence of detectable protein (Watt et al., 2004). We generated a 3′probe, used for Southern blot analysis, by PCR from genomic DNA using the primers g4-192 5′ ATGAAAACAGCTTCCCACCC 3′ and g4-193 5′ AGACTGGCCCTAAGCTATTG 3′. This probe is located between exon 6 and the downstream *SacI* and *EcoRI* sites and identifies 11kb *SacI* (not shown) and 3kb *EcoRI* wild type *Gata4* DNA fragments (Fig. 1a, c). R1 ES cells were electroporated with the pGATA4loxPDT vector and grown in 350μg/ml G418 for 7 days to select for transformants. Homologous recombination was predicted to produce the *Gata4 loxPNeo* allele that generated a unique 5kb *SacI* fragment due to the addition of a *SacI* site within *Neo-tk* and a unique 2kb *EcoRI* fragment due to the addition of an *EcoRI* site in *loxPc* (Watt et al., 2004). We electroporated *Gata4*$^{+/\text{loxPneo}}$ ES cells with a Cre expression plasmid, pHDMCCre8, grew the cells for 3 days without selection, and then cultured in 2μM gancyclovir for 5 days to select for deletion of the *Neo-tk* cassette. Deletion of the *Neo-tk* cassette could occur by recombination between *lox*P*a* and *lox*P*b* (see Fig. 1 of Watt *et al* (Watt et al., 2004)), deleting only the *Neo-tk* cassette and leaving behind a single *lox*P*a/b* site and the *lox*P*c* site flanking exons 3–5. This produces the *Gata4 lox*P allele with an 11kb *SacI* fragment and a 2kb *EcoRI* fragment. Alternatively, recombination between *lox*P a and *lox*P *c* deletes the *neo-tk* cassette and 2kb of genomic DNA leaving behind a single *lox*P a/c site. This produces a *Gata4*$^{-}$ allele with a 9kb *SacI* fragment (not shown) and a 2kb *EcoRI* fragment (Fig. 1a,c) (Watt et al., 2004). *Gata4*$^{-/-}$ ES cells were

generated by targeting *Gata4*$^{+/-}$ ES cells with same targeting vector followed by transient expression of Cre as described above. We confirmed the genotype of *Gata4*$^{-/-}$ ES cells by Southern blot (Fig. 1c) and loss of GATA4 expression by RT-PCR analyses of embryoid bodies (Fig. 1e) and immunohistochemistry on *Gata4*$^{-/-}$ ES cell–derived embryos (Watt et al., 2004). We next targeted the *Gata6* gene in *Gata4*$^{-/-}$ES cells using the targeting vector described by Morrisey *et al* (Morrisey et al., 1998) and used by us previously to generate *Gata6*$^{-/-}$ ES cells (Zhao et al., 2005). This vector contains a *Pgk–Neo* cassette, which replaces exons encoding both zinc fingers and results in a *Gata6* null allele (Morrisey et al., 1998). We electroporated *Gata4*$^{-/-}$ ES cells with the targeting vector and collected colonies that were resistant to growth in G418 (350μg/ml). We next identified *Gata6*$^{+/-}$ ES cells by genomic Southern blot analyses using a probe that flanks exon 5 and identifies a 13kb wild type *Gata6 BamH1* fragment and an 8kb *BamH1* fragment in the targeted allele (Fig. 1b, c). We next cultured *Gata4*$^{-/-}$*Gata6*$^{+/-}$ ES cells in culture medium supplemented with an elevated concentration (1.5mg/ml) of G418, as described (Zhao et al., 2005), and identified *Gata4*$^{-/-}$*Gata6*$^{-/-}$ ES cells by Southern blot analyses (Fig. 1c). We produced embryoid bodies after treating ES cells with Noggin and withdrawing leukemia inhibitory factor following the procedure described by Yuasa *et al* (Yuasa et al., 2005). Embryoid bodies were collected at day 9 after removal of LIF from three independent experiments. We produced embryos directly from ES cells by tetraploid embryo complementation as described elsewhere (Nagy and Rossant, 1993) and considered noon on the day we identified a vaginal plug in surrogate mothers as E0.5.

Oligonucleotide arrays, RT-PCR and real-time qRT-PCR

We collected total RNA from three independent control and *Gata4*$^{-/-}$*Gata6*$^{-/-}$ embryoid body preparations using an RNeasy purification kit (Qiagen). Probes were prepared following Affymetrix protocols. Each sample was hybridized to an individual Affymetrix GeneChip Mouse Genome 2.0 array and data were analyzed using DCHIP Ver. 1.3 software. We carried out semi-quantitative RT-PCR as described previously (Zhao et al., 2005). Real time quantitative RT-PCR was performed using SYBR green incorporation with reactions run on a BioRad iCycler™ following the manufacturer's protocol using empirically optimized primer pairs. All oligonucleotide sequences are available on request, with the exception of proprietary oligonucleotides purchased from Superarray Bioscience Corp that were optimized for qRT-PCR amplification of *Mesp1* (cat#PPM24667A) or *Mesp2* (cat# PPM27883A).

Immunohistochemistry, antibodies, histochemistry and *in situ* hybridization

We collected embryos, fixed them with 4% paraformaldehyde, and stored them in 70% ethanol. We processed embryos for paraffin sections as described previously (Watt et al., 2004; Zhao et al., 2005). We performed immunohistochemistry on either whole embryos or sections following microwave antigen retrieval as we discussed elsewhere (Watt et al., 2004; Zhao et al., 2005). We used the following primary antibodies: anti-smooth muscle actin (Sigma A-2547; 1:800); MF20, anti-myosin heavy chain (Developmental Hybridoma Bank; 1:1000), FoxA1 (C-20, Santa Cruz, sc-6553; 1:400), sarcomeric actin (Sigma A-2172; 1:800), HNF4α (C-19, Santa Cruz, sc-6556; 1:500), CD31/PECAM (CD31, BD Pharmingen #553370; 1:50). Whole mount *in situ* hybridization was performed using digoxigenin–labeled probes generated by *in vitro* transcription (Roche) following standard procedures.

RESULTS

GATA4 and GATA6 are essential for differentiation of cardiac myocytes from ES cells

To test the proposal that GATA4 and GATA6 had redundant yet essential roles in controlling cardiac myocyte differentiation, we generated *Gata4*$^{-/-}$ *Gata6*$^{+/+}$ (Watt et al., 2004), *Gata4*$^{+/+}$ *Gata6*$^{-/-}$ (Zhao et al., 2005), *Gata4*$^{-/-}$ *Gata6*$^{+/-}$, and *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ ES cells

by gene targeting (Fig. 1a–c) (details of targeting are presented in the methods section). ES cells of each genotype were then induced to form cardiac myocytes following the protocol described by Yuasa et al (Yuasa et al., 2005), and the number of beating embryoid bodies, which presumably reflects cardiac myocyte differentiation, was determined by visual inspection. As described previously (Yuasa et al., 2005) this protocol resulted in the efficient generation of beating cells from wild type R1 ES cells with beating observed in >80% of embryoid bodies (Fig. 1d). When either *Gata4* or *Gata6* was disrupted the number of beating embryoid bodies was dramatically reduced, with only 10% of the embryoid bodies containing contractile cells (Fig. 1d). This reduction in the number of beating cells is similar to that previously reported for loss of GATA4 in ES cell embryoid bodies and may reflect a loss of extraembryonic endoderm necessary for maturation rather than a cell autonomous effect (Narita et al., 1997a). No significant difference was observed in the number of beating embryoid bodies when cells lacked either GATA4 or GATA6. However, if one allele of *Gata6* was disrupted in the *Gata4*$^{-/-}$ ES cells then the number of beating embryoid bodies decreased further to 5% (Fig. 1d). Most notably, no beating cells were identified in embryoid bodies lacking both GATA4 and GATA6 (Fig 1d).

If the loss of beating cells reflects a failure in cardiac myocyte differentiation we predicted that cardiac gene expression should be disrupted in *Gata4*$^{-/-}$;*Gata6*$^{-/-}$ embryoid bodies. RT-PCR was therefore performed on control (*Gata4*$^{+/+}$ *Gata6*$^{+/+}$), *Gata4*$^{-/-}$, *Gata6*$^{-/-}$, *Gata4*$^{-/-}$ *Gata6*$^{+/-}$, and *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ embryoid bodies to determine the abundance of a subset of mRNAs encoding proteins whose expression is characteristic of cardiac myocyte differentiation (Fig. 1e). These included cardiac alpha actin, calponin, and alpha and beta myosin heavy and light chains (encoded by *Actc1*, *Cnn1*, *Myh6*, *Myh7*, *Myl2*, *Myl7*, respectively). Previous *in vivo* analyses had demonstrated that loss of either GATA4 or GATA6 had relatively little impact on expression of these key cardiac myocyte mRNAs (Watt et al., 2004;Zhao et al., 2005). Consistent with these analyses and despite the observation that contractility was reduced in both *Gata4*$^{-/-}$ and *Gata6*$^{-/-}$embryoid bodies, cardiac myocyte gene expression was unaffected by loss of either GATA4 or GATA6 alone when compared with control embryoid bodies (Fig. 1e). However, we measured a large reduction in the levels of these cardiac myocyte mRNAs in embryoid bodies generated from *Gata4*$^{-/-}$ *Gata6*$^{+/-}$ ES cells, and they were virtually undetectable in embryoid bodies generated from *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ ES cells (Fig. 1e). These changes in cardiac mRNA levels were confirmed by oligonucleotide array analyses (not shown).

A complex network of cardiac transcription factors is believed to define the differentiated state of cardiac myocytes (Olson and Schneider, 2003; Srivastava and Olson, 2000), and so we next determined the level of mRNAs encoding such transcription factors using qRT-PCR. Figure 1f shows that the level of *Nkx2.5*, *Tbx20*, *Tbx5*, *Mef2c*, *Gata5*, *Hand1*, and *Hand2* mRNAs was substantially reduced in the absence of both GATA4 and 6 compared to wild type cells. Of note, however, the level of *Isl1*, *Mesp1*, and *Mesp2* mRNAs, which are characteristically expressed in cardiac progenitor cells (Cai et al., 2003; Kitajima et al., 2000; Moretti et al., 2006), was not reduced. Cumulatively, these data confirm that GATA4 and GATA6 have redundant functions in controlling cardiac myocyte differentiation and furthermore suggest that they are dispensable for formation of cardiac progenitor cells.

GATA4 and GATA6 are redundant but essential for cardiac development in mouse embryos

The possibility of functional complementation requires that both GATA factors be expressed during the onset of cardiac development in overlapping cell compartments. We therefore performed *in situ* hybridization analyses to detect *Gata4* and *Gata6* mRNAs in developmentally matched embryos. Figure 2a shows that both *Gata4* and *Gata6* mRNAs could be detected in overlapping patterns in cardiac progenitor cells that are present in the cardiac

crescent at E7.5 (early headfold stage), as well as in the nascent heart tube, cardiac outflow and inflow tracts at E8.5 (6–8 somites), which is consistent with previous reports (Dodou et al., 2004; Morrisey et al., 1996; Waldo et al., 2001). We next compared development of the heart in embryos generated from either control or *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ ES cells by tetraploid embryo complementation in order to definitively establish whether these factors were essential for cardiac myocyte differentiation *in vivo*. Figure 2b shows that by E8.5 control wild type ES cell–derived embryos had formed a distinctive heart tube that protruded from the ventral face of the embryo (arrowhead). In contrast, the heart appeared to be completely absent from embryos (n>97) generated from *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ ES cells (Fig. 2b). Acardia was found to occur in embryos generated from three independently targeted *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ ES cell lines and was 100% penetrant. The absence of the heart in GATA4/6 null embryos did not appear to be a consequence of a general embryonic arrest or a defect in gastrulation because histological sections revealed that *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ embryos retained many features characteristic of this developmental stage (Fig. 2c). This included the generation of the expected number of somite pairs (8–12 somite pairs per embryo), the formation of a headfold and the development of the neural tube (Fig. 2c, H&E). The presence of condensed somitic mesoderm confirmed that formation, migration and patterning of the paraxial mesoderm was intact (Fig. 2c, H&E). In addition, the presence of definitive endoderm lining the entire ventral aspect of both control and experimental embryos was confirmed using immunohistochemistry to detect FoxA1 (Fig. 2c, FoxA1) and *in situ* hybridization to detect FoxA2 (Fig. 2c, FoxA2), although it was noted that morphogenesis of the foregut was deficient in the mutant embryos. The possibility that the FoxA1/A2 expressing endoderm was in fact extra embryonic endoderm was excluded because this endoderm failed to express HNF4α (Fig. 2c, HNF4), which is present exclusively in the extra embryonic endoderm at this developmental stage (Duncan et al., 1994; Taraviras et al., 1994). Detection of *Foxa2* mRNA also revealed that the floor plate of the neural tube had been generated in the mutant embryos, although the domain of *Foxa2* expression appeared to be expanded compared with controls. Finally, like control embryos, *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ embryos were found to generate blood vessels that expressed the endothelial cell marker CD31 (Fig. 2c, PECAM). Together, these data demonstrate that loss of both GATA4 and GATA6 has a relatively specific effect on the development of the cardiogenic mesoderm and does not cause a general deficiency in gastrulation or mesodermal migration.

We next performed immunohistochemistry to detect markers of cardiac myocytes to determine whether GATA4 and GATA6 were required for the onset of myocyte differentiation or whether the absence of the heart was primarily due to a block in morphogenesis of differentiated cardiac cells. Figure 3 shows that smooth muscle actin (SMA), alpha myosin heavy chain (αMHC), and alpha cardiac actin (α-sarcomeric actin) could be detected in the differentiating cardiac myocytes forming the heart tube as well as in the yolk sacs of E8.5 (8–12 somites) embryos derived from control ES cells. In contrast, expression of these proteins was undetectable in *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ embryos, although staining was easily identifiable in the yolk sac (Fig. 3). We also examined expression of the co-activator *Smarcd3* (encoding Baf60c) as an example of a gene expressed in myocytes that is not a structural component of myofibrils. *Smarcd3* mRNA was found to be robustly expressed in the cardiac crescent in control embryos, as described previously (Lickert et al., 2004), but was absent from the corresponding region of *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ embryos (Fig. 3, Smarcd3). At about E7.5 (early headfold), cardiac progenitors have formed the cardiac crescent that overlays the anterior intestinal portal, and expression of myocyte markers can first be identified in a subset of cells within the crescent that have committed to a cardiac myocyte cell-fate (Olson and Schneider, 2003). While we identified nascent cardiac myocytes in control embryos using immunohistochemistry to detect smooth muscle actin (Fig. 3, bottom panel, arrowhead), no smooth muscle actin positive cells, outside of the yolk sac, could be detected in *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ embryos (Fig. 3, Sma).

Although transcription factors expressed in cardiac progenitor cells could be detected in *Gata4*$^{-/-}$*Gata6*$^{-/-}$ embryoid bodies, we considered the possibility that the absence of differentiated cardiac myocytes could result from a failure in formation of progenitor cells *in vivo*. We therefore examined the expression of mRNAs encoding cardiac progenitor cell markers *Isl1*, *Nkx2.5*, and *Tbx5*. *Isl1* mRNA, which is predominantly expressed in progenitors within the second heart field (Cai et al., 2003), could be detected in both control and mutant embryos at both E8.5 and E7.5 (Fig. 4, Isl1). Similarly, *Nkx2.5* mRNA, which is expressed in both primary and second heart fields, was also detected in both control and mutant embryos at E8.5 (Fig. 4, Nkx2.5). Both *Isl1* and *Nkx2.5* mRNA were found in tissue sections to be most abundant in two lateral domains of mesoderm that mark the cardiac progenitors (Fig. 4). In contrast to those markers expressed in the second heart field, expression of *Tbx5* mRNA, which is predominantly expressed in the primary heart field progenitor cells and was detectable in control embryos, was undetectable in *Gata4*$^{-/-}$*Gata6*$^{-/-}$ embryos (Fig. 4, Tbx5). These data imply that while GATA4 and GATA6 act to control the onset of cardiac myocyte differentiation they are dispensable for formation of second heart field progenitor cells.

DISCUSSION

In summary, we conclude that GATA4 and GATA6 have overlapping but essential roles in ensuring that progenitors follow a cardiac myocyte cell fate during mammalian development. The mechanism through which this occurs is likely to be complex, and clarity will require further study. However, our data demonstrate that, while formation of the second heart field progenitor cells and their anterior migration to the cardiac crescent is independent of GATA4 or GATA6 function, the differentiation of these progenitors into cardiac myocytes as well as formation of the primary heart field progenitors is blocked by the absence of both GATA4 and GATA6. Such a conclusion is also supported by our analyses of cardiac myocyte differentiation from ES cells in which expression of markers of cardiac myocytes and transcription factors expressed in the primary heart field are dramatically reduced in the absence of GATA4 and GATA6, whereas markers of the second heart field, such as Isl1, or early undefined cardiac progenitor cells, such as Mesp1 and Mesp2, are unaffected by loss of GATA4 and GATA6. In addition to cardiac myocytes, GATA4 and GATA6 are both expressed in the early definitive endoderm, and so the question of whether the dependence on GATA4 and GATA6 for differentiation of cardiac myocytes is strictly cell autonomous has yet to be resolved. Based on the above analyses of *Gata4*$^{-/-}$*Gata6*$^{-/-}$ embryos, we are confident that the definitive endoderm is generated in the absence of GATA4 and GATA6; however, we cannot definitively exclude the possibility that deficiencies in endoderm morphogenesis or differentiation contribute to the absence of cardiac myocytes in these embryos. Aggregation chimeras generated between ROSA26 morulae and *Gata4*$^{-/-}$*Gata6*$^{-/-}$ ES cells did not answer this possibility because in such chimeras both the endoderm and developing heart were populated by wild type cells (not shown). Nevertheless, a significant body of evidence supports the view that although the endoderm is required for later myocardial contraction (Jacobson and Sater, 1988), it is dispensable for the onset of cardiac myocyte differentiation. For example, expression of cardiac differentiation markers in chick lateral plate mesoderm occurs normally following the removal of the definitive endoderm (Gannon and Bader, 1995), and Zebrafish *casanova* mutants, which suffer from a complete absence of definitive endoderm, exhibit cardia bifida but still generate cardiac mycocytes (Alexander et al., 1999). A cell autonomous role for GATA control of cardiac myocyte differentiation is also supported by the observation that the GATA factors, whose own expression responds to BMP signaling (Schultheiss et al., 1997), can directly control transcription of genes encoding numerous cardiac transcription factors and structural proteins, including MEF2c (Dodou et al., 2004) and NKX2.5 (Reecy et al., 1999). Such findings, along with our current analyses, support a model whereby, in response to BMP signaling, the GATA factors act as a molecular lynchpin to ensure robust expression

of the network of transcription factors that orchestrates expression of cardiac myocyte genes during progenitor cell differentiation.

Acknowledgements

We thank R. Misra for RT-PCR primers, E. Morrisey for supplying the *Gata6* targeting vector, B. Brunneau for *Smarcd3* and *Tbx5* and J. Lough for *Nkx2.5* and *Isl1 in situ* hybridization probes, and A.F. Parlow (NHPP) for PMSG. Funding for this project was provided by an American Heart Association fellowship to A.J.W. and National Institutes of Health (NIDDK) grants to S.A.D., as well as gifts from the Marcus Family, Jack and Pheobe Lewis, and the Sophia Wolf Quadracci Memorial Fund.

References

Alexander J, Rothenberg M, Henry GL, Stainier DY. casanova plays an early and essential role in endoderm formation in zebrafish. Dev Biol 1999;215:343–57. [PubMed: 10545242]

Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 2003;5:877–89. [PubMed: 14667410]

Dodou E, Verzi MP, Anderson JP, Xu SM, Black BL. Mef2c is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development. Development 2004;131:3931–42. [PubMed: 15253934]

Duncan SA, Manova K, Chen WS, Hoodless P, Weinstein DC, Bachvarova RF, Darnell JE Jr. Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut, and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. Proc Natl Acad Sci USA 1994;91:7598–7602. [PubMed: 8052626]

Gannon M, Bader D. Initiation of cardiac differentiation occurs in the absence of anterior endoderm. Development 1995;121:2439–50. [PubMed: 7671808]

Holtzinger A, Evans T. Gata4 regulates the formation of multiple organs. Development 2005;132:4005–14. [PubMed: 16079152]

Holtzinger A, Evans T. Gata5 and Gata6 are functionally redundant in zebrafish for specification of cardiomyocytes. Dev Biol 2007;312:613–22. [PubMed: 17950269]

Jacobson AG, Sater AK. Features of embryonic induction. Development 1988;104:341–59. [PubMed: 3076860]

Kitajima S, Takagi A, Inoue T, Saga Y. MesP1 and MesP2 are essential for the development of cardiac mesoderm. Development 2000;127:3215–26. [PubMed: 10887078]

Koutsourakis M, Langeveld A, Patient R, Beddington R, Grosveld F. The transcription factor GATA6 is essential for early extraembryonic development. Development 1999;126:723–32.

Kuo CT, Morrisey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, Soudais C, Leiden JM. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev 1997;11:1048–60. [PubMed: 9136932]

Lickert H, Takeuchi JK, Von Both I, Walls JR, McAuliffe F, Adamson SL, Henkelman RM, Wrana JL, Rossant J, Bruneau BG. Baf60c is essential for function of BAF chromatin remodelling complexes in heart development. Nature 2004;432:107–12. [PubMed: 15525990]

Molkentin JD, Lin Q, Duncan SA, Olson EN. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev 1997;11:1061–72. [PubMed: 9136933]

Molkentin JD, Tymitz KM, Richardson JA, Olson EN. Abnormalities of the genitourinary tract in female mice lacking GATA5. Mol Cell Biol 2000;20:5256–60. [PubMed: 10866681]

Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, Qyang Y, Bu L, Sasaki M, Martin-Puig S, Sun Y, Evans SM, Laugwitz KL, Chien KR. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 2006;127:1151–65. [PubMed: 17123592]

Morrisey EE, Ip HS, Lu MM, Parmacek MS. GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. Dev Biol 1996;177:309–22. [PubMed: 8660897]

Morrisey EE, Ip HS, Tang Z, Parmacek MS. GATA-4 activates transcription via two novel domains that are conserved within the GATA-4/5/6 subfamily. J Biol Chem 1997;272:8515–24. [PubMed: 9079680]

Morrisey EE, Tang Z, Sigrist K, Lu MM, Jiang F, Ip HS, Parmacek MS. GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. Genes Dev 1998;12:3579–90. [PubMed: 9832509]

Nagy, A.; Rossant, J. Production of completely ES cell-derived fetuses. In: Joyner, A., editor. Gene targeting: A practical approach. Oxford Unviersity Press; Oxford, UK: 1993. p. 147-179.

Narita N, Bielinska M, Wilson DB. Cardiomyocyte differentiation by GATA-4-deficient embryonic stem cells. Development 1997a;124:3755–64. [PubMed: 9367431]

Narita N, Bielinska M, Wilson DB. Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. Dev Biol 1997b;189:270–4. [PubMed: 9299119]

Nemer G, Nemer M. Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. Dev Biol 2003;254:131–48. [PubMed: 12606287]

Olson EN, Schneider MD. Sizing up the heart: development redux in disease. Genes Dev 2003;17:1937–56. [PubMed: 12893779]

Peterkin T, Gibson A, Patient R. GATA-6 maintains BMP-4 and Nkx2 expression during cardiomyocyte precursor maturation. Embo J 2003;22:4260–73. [PubMed: 12912923]

Peterkin T, Gibson A, Patient R. Redundancy and evolution of GATA factor requirements in development of the myocardium. Dev Biol 2007;311:623–35. [PubMed: 17869240]

Pikkarainen S, Tokola H, Kerkela R, Ruskoaho H. GATA transcription factors in the developing and adult heart. Cardiovasc Res 2004;63:196–207. [PubMed: 15249177]

Reecy JM, Li X, Yamada M, DeMayo FJ, Newman CS, Harvey RP, Schwartz RJ. Identification of upstream regulatory regions in the heart-expressed homeobox gene Nkx2-5. Development 1999;126:839–49. [PubMed: 9895330]

Reiter JF, Alexander J, Rodaway A, Yelon D, Patient R, Holder N, Stainier DY. Gata5 is required for the development of the heart and endoderm in zebrafish. Genes Dev 1999;13:2983–95. [PubMed: 10580005]

Schultheiss TM, Burch JB, Lassar AB. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Dev 1997;11:451–62. [PubMed: 9042859]

Srivastava D, Olson EN. A genetic blueprint for cardiac development. Nature 2000;407:221–6. [PubMed: 11001064]

Taraviras S, Monaghan AP, Schutz G, Kelsey G. Characterization of the mouse HNF-4 gene and its expression during mouse embryogenesis. Mech Dev 1994;48:67–79. [PubMed: 7873404]

Waldo KL, Kumiski DH, Wallis KT, Stadt HA, Hutson MR, Platt DH, Kirby ML. Conotruncal myocardium arises from a secondary heart field. Development 2001;128:3179–88. [PubMed: 11688566]

Watt AJ, Battle MA, Li J, Duncan SA. GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. Proc Natl Acad Sci U S A 2004;101:12573–8. [PubMed: 15310850]

Yuasa S, Itabashi Y, Koshimizu U, Tanaka T, Sugimura K, Kinoshita M, Hattori F, Fukami S, Shimazaki T, Ogawa S, Okano H, Fukuda K. Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. Nat Biotechnol 2005;23:607–11. [PubMed: 15867910]

Zhao R, Watt AJ, Li J, Luebke-Wheeler J, Morrisey EE, Duncan SA. GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. Mol Cell Biol 2005;25:2622–31. [PubMed: 15767668]

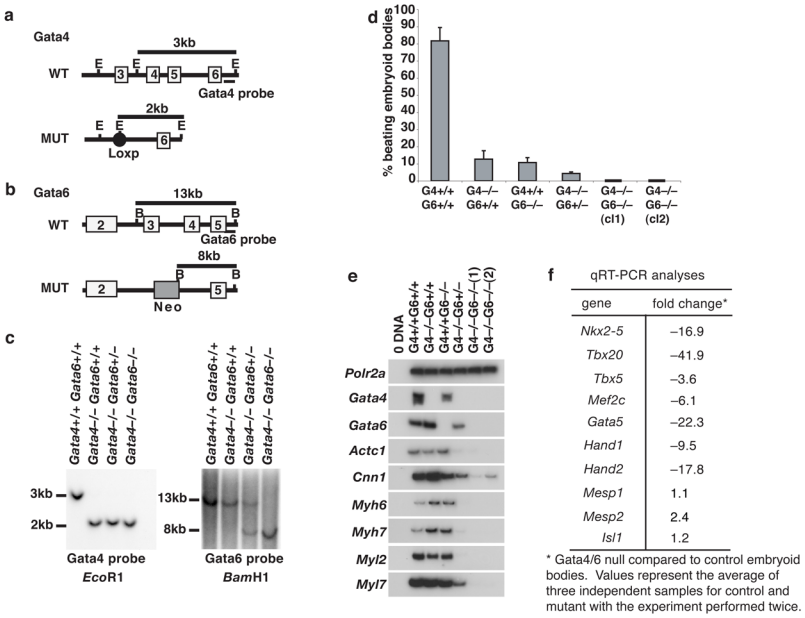

Figure 1. Loss of both GATA4 and GATA6 disrupts cardiac myocyte gene expression in ES cell embryoid bodies

a,**b**, Schematic overview of the strategy used to generate *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ ES cells. Genomic structure of wild type (WT) and mutated (MUT) GATA4 (**a**) and GATA6 (**b**) alleles with exons presented as open boxes, the *lox*P site as a closed circle, and the *Pgk*-Neo cassette as a shaded box. Position of Southern blot probes and sizes of relevant genomic DNA fragments following digestion with EcoRI(E) or BamHI(B) are indicated. **c**, Southern blot confirming the genotype of ES cell lines with DNA fragment sizes shown in kb. **d,** Graph showing percentage of contractile embryoid bodies generated from control (R1) and experimental *Gata4*$^{-/-}$*Gata6*$^{+/+}$, *Gata4*$^{+/+}$ *Gata6*$^{-/-}$, *Gata4*$^{-/-}$ *Gata6*$^{+/-}$, *Gata4*$^{-/-}$ *Gata6*$^{-/-}$ (clones 1 and 2) ES cells in three independent experiments (error bars). **e**, RT-PCR analysis of steady-state cardiac mRNA levels in control and GATA knockout embryoid bodies. *Polr2a; RNA Polymerase II*, *Actc1; cardiac alpha actin*, *Cnn1;Calponin*, *Myh6;cardiac alpha myosin heavy chain*, *Myh7;cardiac beta myosin heavy chain*, *Myl2;cardiac myosin light chain 2v*, *Myl7;myosin light chain 2a*. **f**, Table showing changes, determined by real-time quantitative RT-PCR analyses, in abundance of mRNAs encoding cardiac transcription factors.

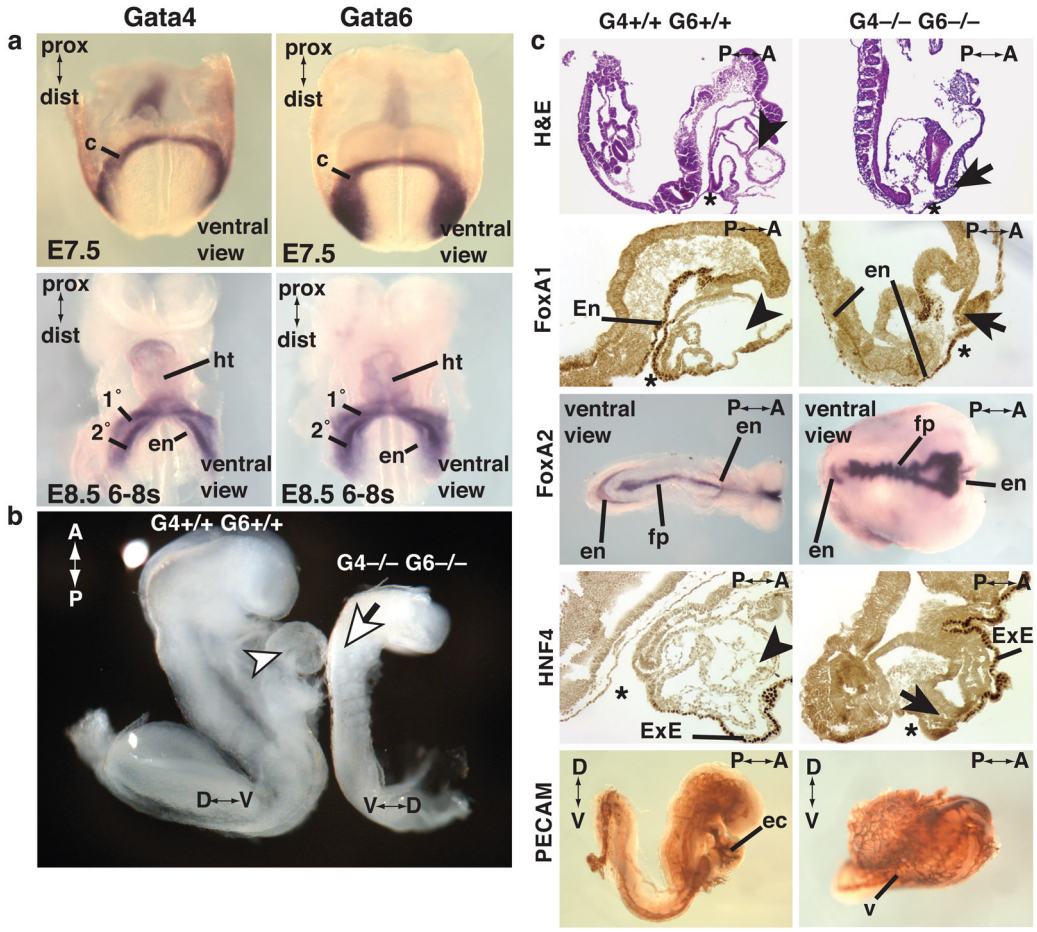

Figure 2. Loss of both GATA4 and GATA6 precludes development of the heart

a, Distribution of *Gata4* and *Gata6* mRNAs detected in E7.5, early headfold stage, and E8.5, 6–8 somite stage, embryos by *in situ* hybridization. **b,** Micrograph showing typical E8.5 embryos generated from either control ($G4^{+/+}$ $G6^{+/+}$) or GATA4/6 doubly null ($G4^{-/-}$ $G6^{-/-}$) ES cells from which the yolk sac has been removed. **c,** Sections of control or GATA4/6 null embryos stained with hematoxylin and eosin (H&E), by immunohistochemistry for expression of FoxA1, HNF4α, or Pecam (CD31), and by *in situ* hybridization for *Foxa2* mRNA. The presence of cardiac tissue in control embryos is indicated with an arrowhead, the expected location of the heart in mutant embryos with an arrow, and the anterior intestinal portal by an asterisk (*). c, cardiac crescent; 1°,presumptive primary heart field; 2° presumptive second heart field; en, definitive endoderm; ht, primary heart tube; fp, neural tube floorplate; ec, endocardium; ExE, extra embryonic endoderm; v, blood vasculature. The positions of the proximal-distal (prox-dist), anterior-posterior (A–P) and dorsal-ventral (D–V) axes are shown where appropriate.

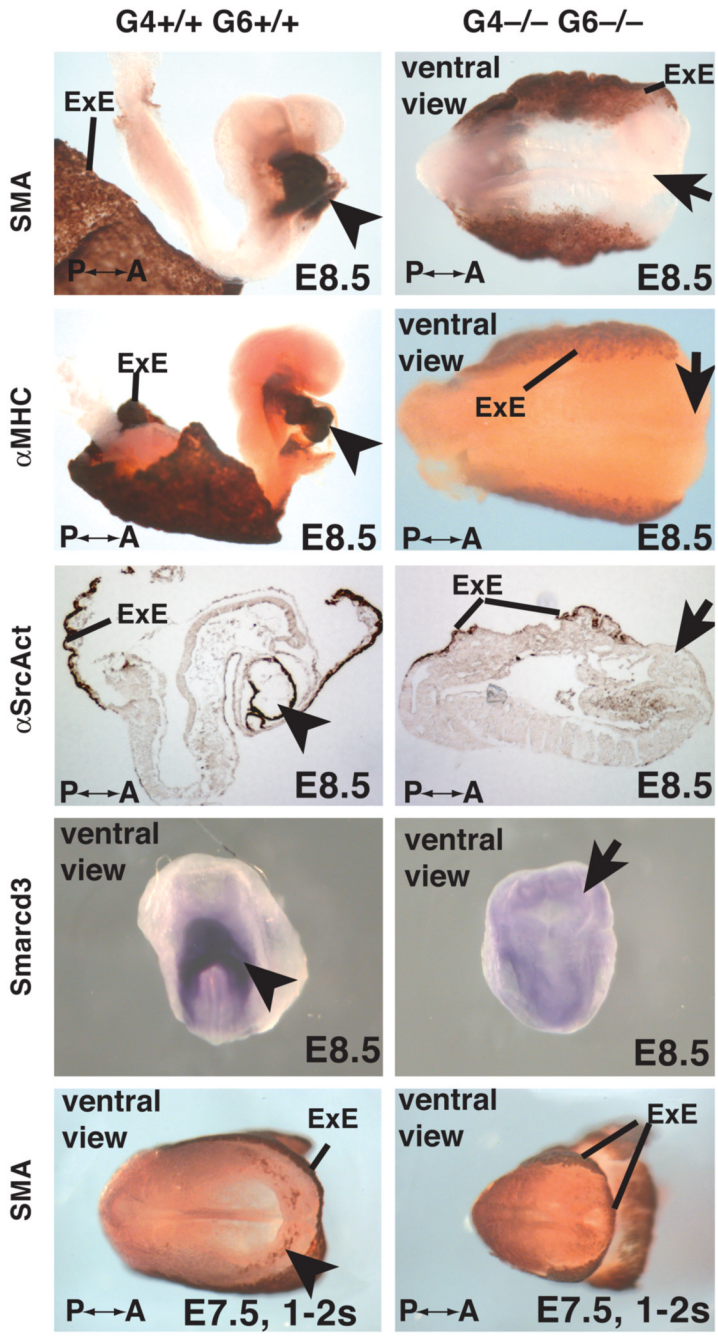

Figure 3. Differentiated cardiac myocytes are undetectable in GATA4/GATA6 null embryos

Micrographs showing control (G4+/+ G6+/+) and mutant (G4−/− G6−/−) whole embryos or sections of embryos at E8.5 (8–10 somites) or E7.5 (1–2 somites) stained by immunohistochemistry (brown staining) to reveal expression of smooth muscle actin (SMA), myosin heavy chain (MHC), sarcomeric actin (αSrcAct), or by *in situ* hybridization to identify *Smarcd3* mRNA. Cardiac myocytes are indicated with an arrowhead in control embryos and the expected position of such cells with an arrow in mutant embryos. Anterior-posterior axis (A-P) and extra-embryonic endoderm (ExE) is shown where appropriate.

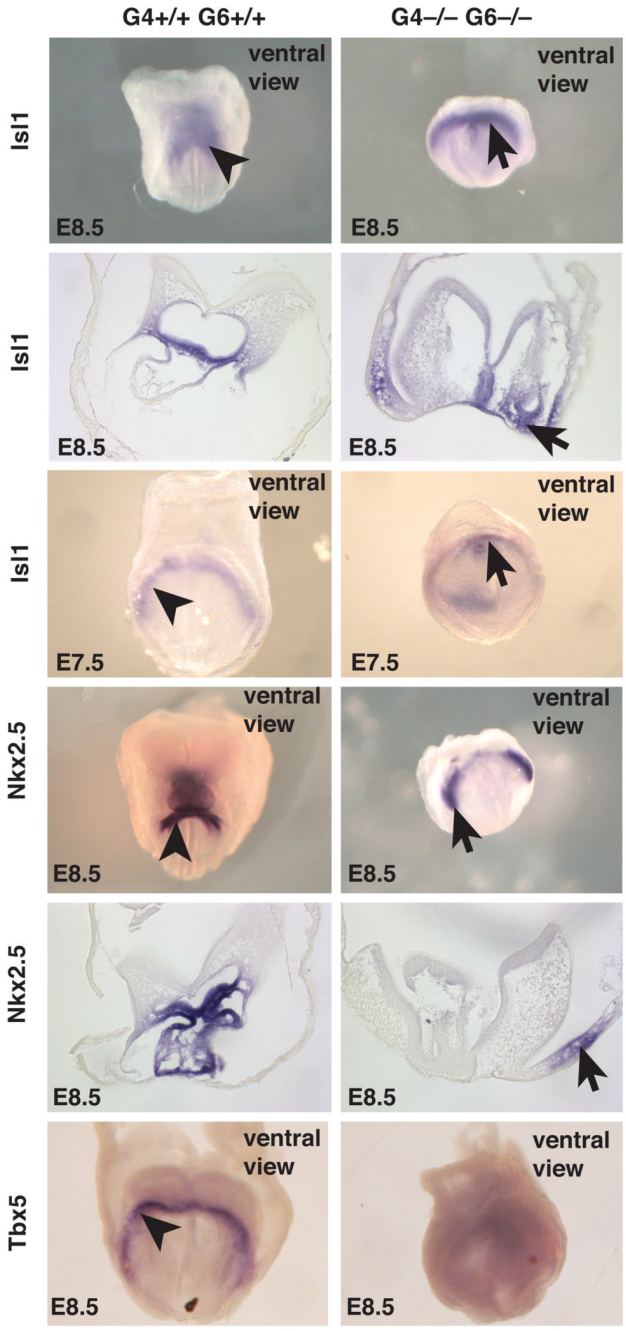

Figure 4. GATA4 and GATA6 are dispensable for formation of second heart field cardiac progenitor cells

Micrographs showing the expression of *Isl1* in E8.5 (8–10 somites) and E7.5 (early headfold) embryos, and *Nkx2.5* and *Tbx5* in E8.5 embryos (8–10 somites) by *in situ* hybridization. Arrowheads and arrows show the presence of presumptive cardiac progenitor cells in control and mutant embryos, respectively. Sections through *Isl1* and *Nkx2.5*–stained embryos confirm the presence of these *mRNAs* in the mesoderm. All panels show ventral views of embryos with the proximal-distal axis positioned from top to bottom.