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Gata4 directs development of cardiac-inducing endoderm from ES cells

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ABSTRACT

The transcription factor *Gata4* is essential for normal heart morphogenesis and regulates the survival, growth, and proliferation of cardiomyocytes. We tested if *Gata4* can specify cardiomyocyte fate from an uncommitted stem or progenitor cell population, by developing a system for conditional expression of *Gata4* in embryonic stem cells. We find that in embryoid body cultures containing even a low ratio of these cells, expression of *Gata4* is sufficient to enhance significantly the generation of cardiomyocytes, via a non-cell-autonomous mechanism. The Gata4-expressing cells do not generate cardiac or other mesoderm derivatives. Rather, *Gata4* expression directs the development of two types of *Sox17*+ endoderm. This includes an *epCam*+*Dpp4*+ subtype of visceral endoderm. In addition, *Gata4* generates similar amounts of *epCam*+ *Dpp4*− definitive endoderm enriched for *Cxcr4*, *FoxA2*, *FoxA3*, *Dlx5* and other characteristic transcripts. Both types of endoderm express cardiac-inducing factors, including WNT antagonists *Dkk1* and *Sfrp5*, although the visceral endoderm subtype has much higher cardiac-inducing activity correlating with relatively enhanced levels of transcripts encoding BMPs. The *Gata4*-expressing cells eventually express differentiation markers showing commitment to liver development, even under conditions that normally support mesoderm development. The results suggest that *Gata4* is capable of specifying endoderm fates that facilitate, with temporal and spatial specificity, the generation of cardiomyocyte progenitors from associated mesoderm.

Introduction

Understanding the transcriptional and signaling programs that specify cardiomyocyte fate from uncommitted progenitors may provide important clues to impact cellular strategies for treating failing or infarcted heart tissue. GATA factors comprise a small family of highly conserved zinc finger transcription factors that play critical roles in the development of the cardiovascular, hematopoietic, digestive, and reproductive systems (Patient and McGhee, 2002). Based on its expression in early precardiac mesoderm, and the phenotypes caused by various gain- and loss-of-function experiments, *Gata4* is considered a key regulator of cardiogenesis (Pikkarainen et al., 2004). Indeed, mutations in the *Gata4* gene cause human congenital cardiomyopathies, including valve and septal defects (Garg et al., 2003; Rajagopal et al., 2007). However, the genetic programs under *Gata4* control are not well understood. This is a challenging problem to address genetically, since *Gata4* expression is not restricted to a specific lineage, there is potential compensation from co-expressed sister genes (*Gata5* and *Gata6*), and it could potentially function either cell-autonomous or non-cell-autonomous with respect to cardiac lineages.

The murine *Gata4* knockout is embryonic lethal, and while there is a heart phenotype, this is secondary due to defects in extra-embryonic endoderm that affect embryonic folding (Kuo et al., 1997; Molkentin et al., 1997). *Gata4* deficient ES cells retain the ability to differentiate into cardiomyocytes *in vitro*, and the analysis of chimeric mice hosting knockout cells proved that *Gata4* is not required for cardiomyocyte differentiation (Narita et al., 1997a). However, Gata4 does function in cardiac cells. Conditional knockout mice, lacking *Gata4* in specified cardiomyocytes, and transgenic mice expressing only 30% of the normal levels of GATA4 in the heart, both display atrioventricular canal defects and a hypoplastic ventricular myocardium. The ventricular hypoplasia is thought to be associated with defects in cardiac morphogenesis and cardiomyocyte proliferation, rather than differentiation (Pu et al., 2004; Zeisberg et al., 2005). Tetraploid complementation was used to rescue the extra-embryonic defects in *Gata4* null embryos, in order to analyze the function of *Gata4* during mouse organogenesis (Watt et al., 2004). These embryos lack proepicardium and show defects in cardiac morphogenesis, including a hypoplastic ventricular myocardium. Likewise, we showed a morphogenetic defect in zebrafish depleted for Gata4 (Holtzinger and Evans, 2005). *Gata4* continues to be required beyond embryogenesis, since heterozygous mutant adult mice are hypersensitive to pressure-induced stress overload (Bisping et al., 2006). In addition to its requirement for epicardial and myocardial function, *Gata4* is also essential in endocardial cells for cushion EMT and valve development (Rivera-Feliciano et al., 2006).

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In sum, *Gata4* is not required for cardiomyocyte specification. However, gain-of-function experiments suggest that *Gata4* encodes sufficient activity to affect cardiomyocyte fate. Forced expression of *Gata4* enhances cardiogenesis during *Xenopus* embryogenesis (Jiang and Evans, 1996) or in P19 embryonal carcinoma cells (Grepin et al., 1997). Furthermore, *Gata4* expression in *Xenopus* ectodermal explants is sufficient to induce differentiation of cardiomyocytes, even after initial commitment of the explants to epidermal fate (Latinkic et al., 2003). *Gata4*-null ES cells generate cardiomyocytes, yet less efficiently compared to wild-type cells (Narita et al., 1997a). In the gain-of-function experiments *Gata4* might direct programs normally regulated by other GATA factors, or the sum total of GATA factors. Regardless of the mechanism, these experiments suggest that *Gata4* is a viable candidate for enhancing cardiogenesis from a progenitor cell population.

Cardiomyocytes are derived from mesoderm, yet their specification during early embryogenesis is dependent on inductive signals from endoderm that develops in close association with presumptive cardiac mesoderm (Foley et al., 2006). Thus, ablation of the anterior endoderm in *Xenopus* embryos results in a loss of myocardium due to a failure of cardiomyocyte specification (Nascone and Mercola, 1995). *Gata4* also regulates growth of endoderm-derived organs, including gut, liver and pancreas in zebrafish (Holtzinger and Evans, 2005), consistent with the analysis of knockout mice rescued by tetraploid complementation (Watt et al., 2007). Wild-type endoderm is sufficient to rescue cardiogenesis in both mouse (Narita et al., 1997b) and chick (Ghatpande et al., 2000) embryos lacking normal *Gata4* expression in the mesoderm. Therefore, *Gata4* functions in both mesoderm and endoderm, consistent with a role in regulating the emerging lineages derived from a common precursor germ-layer, referred to as the mesendoderm (Loose and Patient, 2004).

The embryonic stem (ES) cell system provides an accessible model to study the early stages of murine cardiomyocyte specification, in the context of embryoid body (EB) development. Indeed, a recent study showed that co-culturing murine ES cell-derived EBs with BMP2 and a combination of visceral endoderm-like cells increases significantly the generation of cardiomyocytes (Bin et al., 2006). However, it is not known if *Gata4* is relevant to this or other inductive mechanisms during cardiogenesis. An experimental challenge is the fact that *Gata4* functions in both extra-embryonic and embryonic endoderm. Direct over-expression of *Gata4* in murine ES cells causes them to differentiate into extra-embryonic endoderm (Fujikura et al., 2002; Zhang et al., 2007), precluding investigation of function during EB development. We therefore generated a system to conditionally express *Gata4* in cultured EBs, and evaluate its ability to direct cardiomyocyte fate.

Material and methods

ES cell line derivation and culture

The embryonic stem cell lines AinV15 and AinV18 were described (Kyba et al., 2002) and we used these parental ES cells (referred to generically as AinV cells) to generate the *Gata4*-inducible lines (and they functioned equivalently as controls). The iresEGFP fragment from pIRES2EGFP (Clontech) was isolated following restriction with XhoI and NotI and inserted into the plox vector to generate plox-iresEGFP. A cDNA fragment encoding a flag-tagged *Gata4* open reading frame (*Gata4*ORF) was cloned into the HindIII and XbaI sites of the KS plasmid (Stratagene) to create KS-FLAG-*Gata4*ORF. The FLAG-*Gata4*ORF was then inserted into the XhoI site of plox-iresEGFP (confirming the appropriate orientation). Twenty micrograms of the resulting targeting construct (plox-FLAG-*Gata4*ORF-iresEGFP) was co-electroporated with 20 μg of the pSalk-CRE expression vector into 8×10^6 AinV cells. Stably transfected cells were selected using increasing concentrations of G418 (Cellgro; up to 400 μg/ml). In addition to harvesting pools of clones, individual clonal lines were also selected by limiting dilution in 96-well plates. In each case, proper integration of the transgene was confirmed by PCR analysis of genomic DNA using the following primers: fwd: 5′-CTAGATCTC-GAAGGATCTGGAG-3′; rev: 5′-ATACTTTCTCGGCAGGAGCA-3′.

ES cells were maintained on irradiated mouse embryonic fibroblasts (MEFs) in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% ES-qualified fetal calf serum (Gemini), penicillin, streptomycin, LIF (2% conditioned medium) and 1.5×10^{-4} M monothioglycerol (MTG; Sigma). For the generation of embryoid bodies, ES cells were depleted from MEFs, trypsinized and plated at day 0 at a concentration of 12,000 cells/ml in ethylene oxide treated dishes (VWR Scientific), in Iscove's Modified Dulbecco Medium (IMDM) supplemented with 15% FCS (Atlas), 2 mM L-glutamine (Gibco/BRL), 10% protein-free hybridoma medium (PFHMII; Gibco-BRL), 0.18 mg/ml transferrin (Roche), 50 ng/ml ascorbic acid (Sigma), 4.5×10^{-4} M MTG, penicillin, and streptomycin. Transgene expression was induced by addition of doxycycline (Sigma) into the culture media at a final concentration of 1 μg/ml.

Western blotting

Total protein extract (20 μg) derived from embryoid bodies was electrophoresed through a 10% NuPAGE Bis-Tris gel using the XCell SureLock Mini-Cell system (Invitrogen) and transferred to a PVDF nylon membrane (Biorad). For detection of Flag-tagged protein, membranes were incubated with an anti-FLAG M2-HRP conjugated monoclonal antibody (Sigma) at a 1:1000 dilution in blocking buffer. For GATA4 detection, membranes were incubated with a rabbit anti-mouse GATA4 primary antibody (Sigma) at a 1:500 dilution, and an HRP-goat anti-rabbit secondary antibody (Biorad) at a 1:10,000 dilution in blocking buffer. Specific bands were detected using the Western Blotting Luminol Reagent (Santa Cruz). To confirm equal loading, gels were stained in Coomassie Blue.

Gel mobility shift assays

Oligonucleotides used as probe or cold competitors were PAGE purified. The upper strand oligomer was end-labeled with [γ-^{32}P]ATP, purified through a G-25 Sephadex column (Roche), and annealed to its complementary lower strand. The competitors were prepared identically, without labeling. The specific competitor was the unlabeled probe. The non-specific competitor contains mutations in the GATA-binding sites. The sequences of the top strands are as follows: probe and specific competitor: 5′-AGCTTGCG*GATAAGATAA*-GGCCGGAATTCA-3′; non-specific competitor: 5′-AGCTTGCGCTGAA-CTGAAGGCCGGAATTCA-3′. Binding reactions and mobility shift assays were carried out as described (Evans et al., 1988).

Differentiation assays

For evaluating cardiomyocyte differentiation, EBs were harvested at day 6 and plated onto gelatin-coated dishes in IMDM supplemented with 2 mM L-glutamine, 10% PFHMII, 0.18 mg/ml transferrin, 50 ng/ml ascorbic acid, 4.5×10^{-4} M MTG, penicillin, and streptomycin. At day 10, the EBs were evaluated visually in blinded samples to quantify the number of beating clusters per total number of EBs. For titration assays, EBs were derived from AinV and iGATA4 ESCs in the following ratios: 1:0, 1:1, 1:3, 1:10, and 1:50. EBs were subsequently processed as described above.

For evaluating hematopoiesis, cells were isolated from EBs at day 6 of development and were plated at a concentration of 100,000 cells/ml into 1% methylcellulose supplemented with 15% plasma-derived serum (PDS; Antech), 5% PFHMII, 2 mM L-glutamine, 0.18 mg/ml transferrin, 25 ng/ml ascorbic acid, 4.5×10^{-4} M MTG, 4 U/ml