GATA factors efficiently direct cardiac fate from embryonic stem cells

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SUMMARY
The GATA4 transcription factor is implicated in promoting cardiogenesis in combination with other factors, including TBX5, MEF2C and BAF60C. However, when expressed in embryonic stem cells (ESCs), GATA4 was shown to promote endoderm, not cardiac mesoderm. The capacity of related GATA factors to promote cardiogenesis is untested. We found that expression of the highly related gene, Gata5, very efficiently promotes cardiomyocyte fate from murine ESCs. Gata5 directs development of beating sheets of cells that express cardiac troponin T and show a full range of action potential morphologies that are responsive to pharmacological stimulation. We discovered that by removing serum from the culture conditions, GATA4 and GATA6 are each also able to efficiently promote cardiogenesis in ESC derivatives, with some distinctions. Thus, GATA factors can function in ESC derivatives upstream of other cardiac transcription factors to direct the efficient generation of cardiomyocytes.

KEY WORDS: Directed differentiation, Cardiomyocyte, Transcription factor, Mesoderm

INTRODUCTION
The transcription factors required for cardiogenesis during normal development include members of the NKX2, MEF2, HAND, SRF, TBX and GATA transcription factor families. These proteins regulate each other, functioning through stabilization of cardogenic transcriptional networks (Olson, 2006). Forced expression of combinations of these transcription factors can direct differentiation of cardiac fate. In particular, Gata4 has been implicated, as this gene is essential for normal embryonic myocardial growth (Holtzinger and Evans, 2005; Rojas et al., 2008), and embryos lacking both Gata4 and the sister gene Gata6 display acardia (Zhao et al., 2008). Forced expression of Gata4 with Tbx5 and the chromatin modulator Baf60c (Smarcd3 – Mouse Genome Informatics), can reprogram somatic mesoderm to cardiac fate (Takeuchi and Bruneau, 2009), and combined expression of Gata4 with Tbx5 and Mef2c was shown to reprogram fibroblasts to cardiomyocytes (Ieda et al., 2010). Yet cardiac reprogramming is inefficient, not highly reproducible (Chen et al., 2012) and has not been adapted to the embryonic stem cell (ESC) system, which can be used for generating unlimited cardiac cells (Murry and Keller, 2008). Expression of Gata4 in ESCs drives development of primitive endoderm, not cardiac cells (Fujikura et al., 2002). We found previously that expression of Gata4 in a defined window of ESC-derived embryoid body (EB) development can enhance production of cardiac cells, but it does so indirectly by inducing the formation of cardiac-promoting endoderm (Holtzinger et al., 2010).

The Gata5 gene is under-studied compared with Gata4 or Gata6, because the Gata5-null embryo does not display a cardiac phenotype (Molkentin et al., 2000). However, in addition to bifida, some gata5/faust zebrafish embryos showed a relative loss of cardiomyocytes (Reiter et al., 1999). Subsequent studies in zebrafish and Xenopus showed that gata5 has an essential but redundant role (with gata6) in cardiac progenitor specification (Holtzinger and Evans, 2007; Haworth et al., 2008). Overexpression of gata5 in combination with smarcd3b generates ectopic cardiac tissue in developing zebrafish embryos (Lou et al., 2011). In comparison with fish, mouse embryos apparently better compensate for loss of Gata5. However, Gata4/5 compound mutant mice display progressively severe cardiac defects (thinned ventricular wall, hypoplastic compact myocardium, abnormal trabecular structures and endocardial cushion defects) secondary to poor myocardial cell proliferation (Singh et al., 2010). Gata4/5 and Gata5/6 compound heterozygotes present outflow tract defects that include double outlet right ventricle and ventricular septal defects (Laforest and Nemer, 2011). Here, we tested the capacity for Gata5 to program cardiac fate in murine ESC derivatives. Using an engineered ESC line that allows conditional expression of Gata5, we found that expression of this single factor efficiently promotes abundant populations of beating and phenotypically normal cardiac cells. This led us to investigate more closely why Gata4 failed in previous studies to direct cardiac fate. We found that, under defined culture conditions, Gata4 (and the other sister gene, Gata6) are capable of similar, but not identical, cardiocommitting capacities.

MATERIALS AND METHODS
ESC line derivation and culture
The murine ESC line AinV18 (Kyba et al., 2002) was used to derive and validate lines that allow conditional expression of Gata5 or Gata6, essentially as described previously (Holtzinger et al., 2010), and were maintained without feeders on gelatin in serum-free conditions consisting of DMEM/F12 and Neurobasal media (Invitrogen) supplemented with N2 (Gibco), B27 (Gibco), BSA (Gibco), penicillin/streptomycin, 2% LIF, 3 mM CHIR 99021 (STEMGENT), 1 mM PD0325901 (STEMGENT), 1.5×10⁻⁴ M MTG (Sigma) and 2 mM L-glutamine (Gibco/BRL).

Embryoid body generation and cell differentiation
ESCs were trypsinized and plated (day 0) at 9000 cells/ml in differentiation media composed of GMEM (Gibco) supplemented with 1 mM sodium
pyruvate (Gibco), 0.1 mM β-mercaptoethanol (Sigma), 5% KOSR (Invitrogen) and 0.1 mM non-essential amino acids (Gibco) (Honda et al., 2006). Media was refreshed on day 4. Transgene expression was induced by the one time addition of doxycycline (Sigma) at 1 μg/ml on day 4. EBs were harvested on day 6 and replated to evaluate cardiomyocyte differentiation on gelatin-coated polystyrene dishes (BD Falcon) in IMDM supplemented with 10% protein-free hybridoma medium (Gibco/Invitrogen), 2 mM L-glutamine (Gibco/BRL), 0.18 mg/ml transferrin (Roche), 50 mg/ml ascorbic acid (Sigma) and 4.5×10⁻⁸ M MTG. In some experiments, EBs were harvested and trypsinized at day 6 and plated as a monolayer on human fibronectin in the above media. Affymetrix microarray transcript profiles from day 16 differentiated cells (deposited with GEO Accession Number GSE43831) were subjected to unsupervised hierarchical cluster analysis using GenePattern 2.0 (Broad Institute, Cambridge, MA) (Eisen et al., 1998; Reich et al., 2006) with microarray data downloaded from GEO listed in supplementary material Table S1. For sorting KDR and PDGFRα populations, cells were cultured in serum-free conditions based on protocols described previously (Kattman et al., 2011). Hematopoietic progenitors were scored in colony assays as described previously (Zafonte et al., 2007).

**Quantitative real-time PCR**

The primers used are listed in supplementary material Table S2. Ct values were calculated using the ΔΔCt method (Livak and Schmittgen, 2001), based on the median value from a triplicate set. Each value was normalized to levels of Gapdh transcripts. Statistical significance was determined using a two-tailed Student's t-test (P<0.05).

**Flow cytometry and immunohistochemistry**

EBs were dissociated and fixed with 2% paraformaldehyde for intracellular staining. Antibodies are listed in supplementary material Table S3. Nonspecific staining was excluded using appropriate isotype control antibodies. Approximately 50,000 single live cell events were recorded and analyzed per sample on a C6 flow cytometer (Accuri) and analyzed using FCS express (De Novo Software). For immunohistochemistry, EBs were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton. Nuclear staining was performed with DAPI (Invitrogen).

**Electrophysiology**

EBs were trypsinized at day 6 and plated as a monolayer on human fibronectin. Day 12 cells were trypsinized and plated onto laminin-coated coverslips for patch clamp studies within 24 to 48 hours. Coverslips were transferred into a recording chamber superfused with normal Tyrode’s solution containing (in mM) NaCl (137.7), KCl (2.3), CaCl2 (1.8), MgCl2 (1), glucose (10) and HEPES (10) (pH adjusted to 7.4 with NaOH). Electrodes were filled with (in mM): KCl (50), K-aspartic acid (80), MgCl2 (1), EGTA (10), HEPES (10) and Na₂-ATP (3) (pH adjusted to 7.2 with KOH). The resistances of the electrodes were between 2 and 3 MΩ. Only cells with gigaseal were used to collect data under current clamp mode at room temperature. Stimulated action potentials were triggered by minimum positive pulses with 1 Hz frequency. Signals were recorded by amplifiers (MultiClamp 700B, Axon Instruments) and digitized (Model DIGIDATA 1440A, Axon Instruments). Data acquisition and analysis were performed using CLAMPPEX 10.2 and CLAMPFIT 10.2 software (Axon Instruments), respectively.

**Ca²⁺ transients with microfluorimetry**

Cells were exposed to a dye-loading solution consisting of a standard Tyrodes containing (in mM): NaCl (140), KCl (4), CaCl2 (2), MgCl2 (1), HEPES (10) and glucose (5.6), pH was adjusted to 7.4 with NaOH at 22°C. The dye-loading solution was supplemented with 2.5 μM Fluo-4 acetoxyethyl ester (Fluo-4/AM; Invitrogen, Eugene, OR, USA). Cells were exposed to Fluo-4/AM for 15 minutes at 22°C. Cells were washed twice and equilibrated in fresh Tyrodes solution for 30 minutes to allow de-esterification of the dye before recording. Fluorescent signals were acquired using a 40x UVF objective (numerical aperture 1.0, Nikon), and single excitation wavelength microfluorimetry was performed using a PMT system (IonOptix, Milton, MA, USA). The Fluo-4-loaded cells were illuminated (excitation filter central wavelength 480 nm) and the fluorescent signal (filter wavelength >505 nm) was collected by a PMT detector.

**RESULTS AND DISCUSSION**

**Conditional expression of Gata5 in a mouse embryonic stem cell line**

As Gata4 expression in ESCs generated endoderm (Fujikura et al., 2002; Holtzinger et al., 2010), we created for comparison analogous murine ESC lines in which Gata5 is placed into the identical doxycycline-inducible locus. The parental AinV cell line (Kyba et al., 2002) expresses constitutively the inactive reverse tetracycline transactivator protein. A cDNA encoding flag-tagged mouse Gata5 was recombined into the pre-targeted loxP site with an IRES-EGFP cassette (supplementary material Fig. S1A). Site-specific integration of the transgene was confirmed by PCR (supplementary material Fig. S1B). Cell lines carrying the transgene are referred to as iGata5ES cells. We demonstrated by western blotting expression of GATA5 upon induction of iGata5ES cells (supplementary material Fig. S1C). GFP co-expression is seen throughout EBs following addition of doxycycline (supplementary material Fig. S1D). By 48 hours, at least 90% of the cells were GFP⁺ (supplementary material Fig. S1E). Several independent iGata5ES clonal lines were adapted to serum-free culture, and they behaved equivalently regarding all phenotypes we describe below.

**Gata5 is capable of promoting cardiogenesis in a serum-free culture system**

In spontaneously differentiating EBs, cardiac progenitors are specified at approximately day 4 of culture (Kattman et al., 2006). Results from a representative quantitative real-time RT-PCR (qPCR) experiment (Fig. 1A) demonstrate that induction of Gata5 at day 4 results by day 6 in a substantial (50- to 1000-fold) enhancement of transcript levels for the early cardiac markers Nkx2.5, Tbx5 and Gata4, compared with uninduced controls. There was no increase in endodermal, ectodermal or hematopoietic markers. The cardiogenic effect was seen when Gata5 was induced at any stage of EB development (Fig. 1B). However, the response was most significant at day 4, which suggests that Gata5 impacts development around the time when mesoderm progenitors commit to a cardiac fate. We evaluated expression of markers for mesodermal derivatives at later developmental time points, including days 6, 10 and 14. Gata5 expression results in highly significant increases, up to 1000-fold in transcript levels, for the cardiac markers Tnn1 (previously cTnT), Myl7 (previously Mlc2a) and Myl2 (previously Mlc2v), compared with uninduced controls (Fig. 1C), and to a lesser extent increased expression of endothelial markers Cdh5 (previously Vecad) and Pecam1 (previously Cd31). Expression of Gata5 did not increase levels of brachyury (Bry) or Mesp1 (rather they were suppressed), whereas transcript levels of Tbx5, Mef2c, Nkx2.5, Isl1 and Gata6 were all markedly enhanced (Fig. 1D). This suggests that Gata5 expands cardiac rather than mesoderm fate.

**Gata5 induction directs the development of cardiomyocytes**

When plated onto gelatin-coated wells, essentially all Gata5-induced EBs display extensive contractile activity beginning as early as day 6 of EB development and persisting through at least day 25 (not shown). When dissociated and plated onto fibronectin, the EB-derived cells form by day 9 extensive sheets of beating tissues (supplementary material Movie 1). Gata5 expression results in a ~10-fold increase in the percentage of cells that stain positively for TNNT2 when compared with uninduced controls, comprising at least half of the population (Fig. 1E,F). When plated as a monolayer, cells that had been induced at day 4 were TNNT2 positive by day 12 (Fig. 1G). We found in the cultures few
CXCR4/EPCAM double-positive endoderm or PECAM1-positive endothelial cells (supplementary material Fig. S2A,B). Approximately 7% of the cells expressed both the smooth muscle cell marker ACTA2 (SMA) and the cardiac marker TNNT2 (Fig. 1H). Immunohistochemistry confirmed the presence of mostly TNNT2+ clusters, some ACTA2 + clusters and occasional TNNT2+/ACTA2+ double-positive clusters in Gata5-induced EB derivatives alone (supplementary material Fig. S2C). Most of the Gata5-directed cells co-expressed TNNT2 and MYL7 (supplementary material Fig. S2D). These phenotypes (TNNT2+/MYL7+, few TNNT2+/ACTA2+, PECAM+) are consistent with previously published studies regarding ESC-derived cardiac cell populations (Kattman et al., 2011). Gata5-induced cultures were harvested at day 16 of differentiation and RNA was profiled by microarray analysis. Unsupervised hierarchical clustering showed these cells are most similar to growth factor-directed ESC-derived cardiac cells (supplementary material Fig. S3). In mixed cultures, increasing the ratio of iGata5ES cells to parental AinV cells resulted in a proportional increase in expression for the cardiac program (supplementary material Fig. S4). As Gata5-induced cardiac cells, but not mesoderm or endoderm, the results suggest that Gata5 promotes in a cell-autonomous manner cardiac fate in ESC-derived mesoderm.

Patch-clamping experiments confirmed that the cardiac cells are electrically active, showing a range of electrophysiological profiles. From 40 randomly selected cells, 35% (14) demonstrated spontaneous action potentials. Based upon resting membrane potential, APD_{50} and APD_{90}/APD_{50} values, nine of these were considered immature cells (Fig. 2A, Table 1) and five demonstrated nodal action potentials (Fig. 2B, Table 1). The remaining 65% (26) of cells were quiescent at baseline. Of these, 19 were classified as atrial (Fig. 2C, Table 1) and seven as ventricular (Fig. 2D, Table 1). Thus, all major subtypes of cardiomyocytes were detected. Calcium fluorimetry on Gata5-induced cardiac cells assessed Ca^{2+} transients and chronotropic responses to adrenergic and muscarinic stimulation. Regular, repetitive and spontaneous calcium oscillations and cell beat length changes were imaged in Gata5-directed cardiac cells (supplementary material Fig. S5A). β-Adrenoceptor stimulation with isoproterenol increased the rate of Ca^{2+} oscillation compared with control, and the muscarinic agonist acetylcholine decreased the rate (Fig. 2E; supplementary material Fig. S5B). Both the cell beat rate (Fig. 2F) and the decay time constant τ (ms) of Ca^{2+} signals (Fig. 2G) demonstrated statistically significant changes with either isoproterenol or acetylcholine. Thus, Gata5-induced cardiac cells display normal Ca^{2+} handling and chronotropic responsiveness.

**Gata5 induction directs mesoderm-committed progenitors to a cardiac fate**

Previous work showed that cardiac progenitors are highly enriched within the KDR (previously FLK1)^+ /PDGFRα^+ cell population.
Table 1. Action potential diversity in iGATA5-derived cardiomyocytes

<table>
<thead>
<tr>
<th>Cell type (n)</th>
<th>RP</th>
<th>APA</th>
<th>APD50</th>
<th>APD90</th>
<th>APD90/APD50</th>
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<tr>
<td>Spontaneously beating cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Immature (9)</td>
<td>–65.7±1.2</td>
<td>85.7±2.7</td>
<td>216.8±36.2*</td>
<td>330.7±44.2</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td>Nodal (5)</td>
<td>–51.2±4.7*</td>
<td>63.6±4.2*</td>
<td>90.4±13.6*</td>
<td>253.1±17.9</td>
<td>3.0±0.4*</td>
</tr>
<tr>
<td>Quiescent cells</td>
<td></td>
<td></td>
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<tr>
<td>Atrial (19)</td>
<td>–63.4±1.3</td>
<td>96.6±2.7</td>
<td>61.2±6.9</td>
<td>195.5±16.6</td>
<td>3.5±0.3</td>
</tr>
<tr>
<td>Ventricular (7)</td>
<td>–71.1±2.0*</td>
<td>98.3±6.4</td>
<td>182.4±47.5*</td>
<td>264.0±64.5</td>
<td>1.5±0.1*</td>
</tr>
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</table>

Comparison of action potential diversity in the four subtypes. RP, resting potential; APA, action potential amplitude; APD50, action potential duration at 50% repolarization; APD90, action potential duration at 90% repolarization. *P<0.05. The number of cells is indicated in parentheses.
Gata4 increases expression of Gata5 less than fivefold. Gata6 fails at this early stage to activate expression of Gata5 (Fig. 4B). Microarray experiments were performed in three independent experiments to profile fully the programs activated by each GATA factor. Comparison of the genes activated or repressed at least fivefold 1 day after induction with doxycycline shows overlap in the differentiation programs directed by each factor (supplementary material Fig. S7A), and gene ontology analysis of this overlap using DAVID confirmed that this gene set is highly enriched in cardiac/muscle genes (supplementary material Fig. S7B).

Fig. 3. Gata5-induced cardiac cells can be generated from KDR+ mesoderm. (A) EB derivatives were sorted at day 4 to isolate by FACS the KDR+/PDGFRα+; KDR+/PDGFRα−; KDR−/PDGFRα+; and KDR−/PDGFRα− populations. A representative plot is shown. (B) Cells purified from each of the gates were reaggregated (P−, PDGFRα−; K+, KDR+), induced with doxycycline plated at day 6 and scored at day 12 for beating colonies, compared with the same samples that were left uninduced. Data are from three independent experiments and asterisks indicate significant differences between uninduced and induced samples (*P<0.05; **P<0.01). Significance for P+ could not be determined because the uninduced cells do not form any beating colonies. (C) Immunohistochemistry of the samples at day 18 shows representative fields of TNNT2+ cells (red). In the uninduced samples, these are seen only in unsorted and P+/K+ cells. By contrast, Gata5-expressing cells (Dox) generate abundant TNNT2+ cells in each population, but especially from K+ sorts.

Fig. 4. Each of the GATA factors, under serum-free conditions, has capacity to induce cardiogenesis, but with distinctions. (A) Each line, iGata4ES (blue), iGata5ES (red) or iGata6ES (green) was induced at day 4 and RNA analyzed for the indicated cardiac genes (Tbx5, Mef2c, Nkx2.5 or Isl1) or mesoderm gene (Bry, Pdgfra, Kdr or Mesp1) by qPCR at day 5, compared with each uninduced control. The fold change is shown from three independent experiments. Statistical significance is indicated: *P<0.05, **P<0.01 or ***P<0.001. (B) Data taken from the same samples as A, showing comparison of crosstalk among the GATA genes themselves, plotted as log differences compared with the uninduced controls for iGata4ES (IG4), iGata5ES (IG5) or iGata6ES (IG6). (C) Each of the lines was induced at day 4, EBs were plated for cardiac differentiation, and RNA was harvested at day 6 and evaluated for expression of cardiac genes by qPCR. Shown are the results from three independent experiments with relative fold change compared with uninduced samples graphed as log2 values.
When EBs were induced with doxycycline to express each GATA factor at day 4, and plated for differentiation, all three lines generated abundant beating structures by day 6 (not shown). Comparison of the differentiating cells at this stage showed for each line a similarly robust activation of a cardiogenic program compared with each uninduced control (Fig. 4C). Although Isl1 levels were decreased, the other regulatory genes and cardiac structural genes were highly expressed. Again, there are some distinctions, as Gata6 expression was most effective. In contrast to the other two genes, Gata6 activates expression of FoxC2, suggesting some distinct cardiac cell types in the Gata6-programmed culture (Kunne, 2009).

ESC s provide a promising platform for the development of therapeutic strategies and drug testing (Burridge et al., 2012). Here, we showed that conditional expression of a single GATA factor is sufficient to highly expand cardiac cells in an ESC system, dependent on removing suppressive factors present in serum. Another gene with cardiac-inducing activity in ESCs is the bHLH transcription factor Mespl (David et al., 2008; Bondue and Blanpain, 2010). Consistent with its normal expression pattern in a wider mesodermal domain, Mespl may promote a broader set of lateral plate derivatives, and also neural ectoderm, whereas GATA factors direct cardiac cells from KDR+ mesoderm, consistent with their function in a developmental network downstream of Mespl and upstream of Tbx5, Nkx2.5 and Mef2c.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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