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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 cardiogenic 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 *cardia 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 Gata*5/6* 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, cardiac-promoting 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

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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 ng/ml ascorbic acid (Sigma) and 4.5×10^{-4} 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. C_t values were calculated using the $\Delta\Delta C_t$ 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, EB derivatives 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 (5.4), NaOH (2.3), $CaCl_2$ (1.8), $MgCl_2$ (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), $MgCl_2$ (1), EGTA (10), HEPES (10) and Na_2-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 CLAMPEX 10.2 and CLAMFIT 10.2 software (Axon Instruments), respectively.

Ca^{2+} transients with microfluorimetry

Cells were exposed to a dye-loading solution consisting of a standard Tyrodes containing (in mM): NaCl (140), KCl (4), $CaCl_2$ (2), MgCl (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 acetoxymethyl 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 40× 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 *Tnnt2* (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

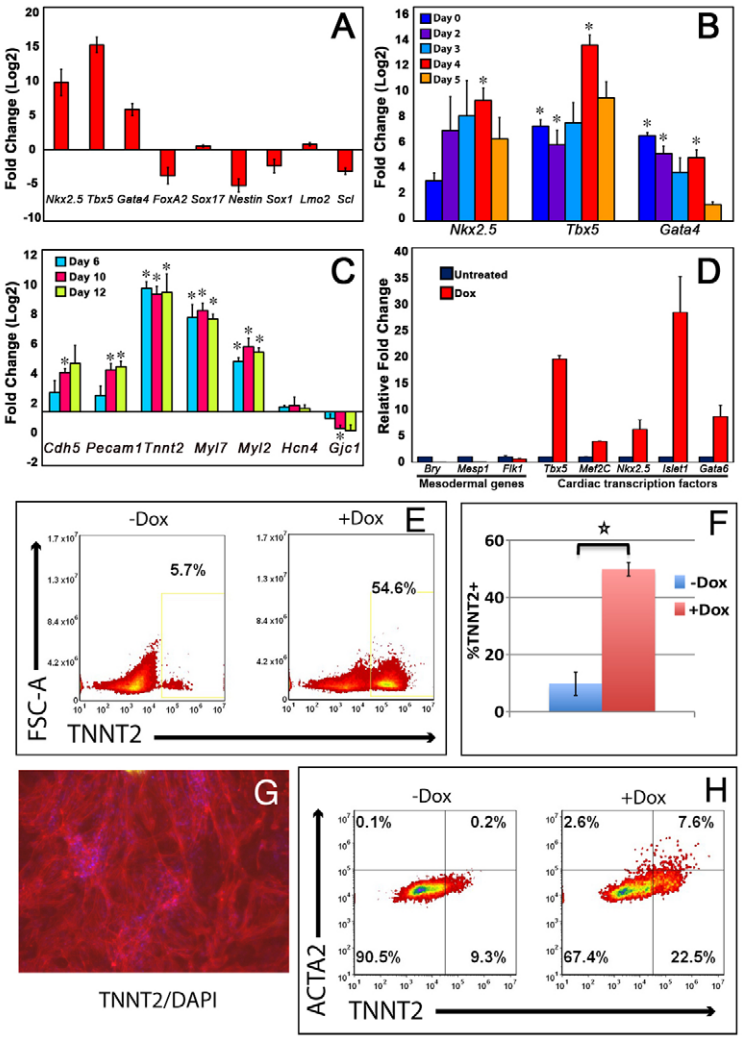

Fig. 1. Induction of *Gata5* expression in EBs robustly promotes cardiogenesis. (**A**) Representative qPCR analysis from three independent experiments with RNA isolated from day 6 EBs following *Gata5* induction at day 4, normalized to levels in day 6 uninduced controls. Fold change is log2 transformed for the markers indicated. Error bars indicate s.e.m. (**B**) qPCR analysis for early cardiac regulatory genes in day 6 EBs following *Gata5* induction at days 0, 2, 3, 4 or 5, normalized to levels in uninduced controls. Results are from three independent experiments. Error bars show s.e.m.; **P*<0.05. (**C**) qPCR analysis for differentiation markers as indicated with RNA isolated from day 6, day 10 or day 14 EBs following *Gata5* induction at day 4, plotted as in B. (**D**) qPCR analysis for expression of a wider range of mesoderm or cardiac regulatory genes in day 5 EBs following *Gata5* induction at day 4. Cardiac genes are enhanced and mesoderm markers repressed; all levels in induced EBs are significantly different from the uninduced samples (*P*<0.05). Error bars indicate s.e.m. (**E**) EBs induced on day 4 were analyzed by flow cytometry for TNNT2 on day 12. (**F**) Summary of flow cytometry results from 10 independent experiments for TNNT2$^+$ cells in 12 day EBs that had been induced on day 4. Data are mean±s.e.m. Star indicates *P*<0.01. (**G**) Induced EB derivatives plated onto fibronectin were stained for TNNT2 at day 12 and counterstained with DAPI. (**H**) Induced EB derivatives were analyzed for TNNT2 and ACTA2 on day 12.

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

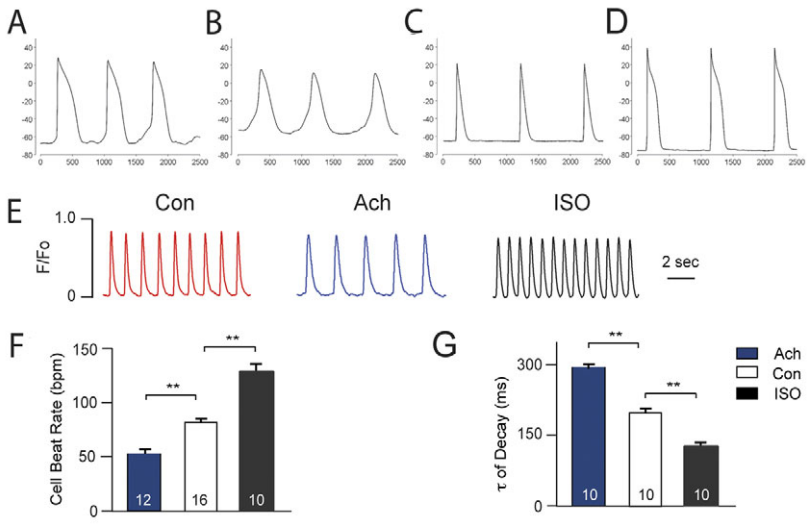

Fig. 2. *Gata5*-directed cardiac cells display a full range of electrophysiological profiles with adrenergic and muscarinic responsiveness. (**A-D**) Four major types of action potential were identified based on morphology. (A) Immature, (B) pacemaker, (C) atrial and (D) ventricular. (**E**) Shown are spontaneous Ca^{2+} oscillations and following addition of 10 μM acetylcholine (Ach) or 1 μM isoproterenol (Iso). (**F**,**G**) Cell beat rate (beats per minute, bpm) (F) and decay time constant τ (ms) (G) of Ca^{2+} signals. Data are means±s.e.m. (**P<0.01).

(Hirata et al., 2007; Kattman et al., 2011). EBs were harvested and dissociated at day 2 and reaggregated under conditions that generate distinct KDR$^+$/PDGFRα$^+$, KDR$^+$/PDGFRα$^-$, KDR$^-$/PDGFRα$^+$ and KDR$^-$/PDGFRα$^-$ populations (Kattman et al., 2011). Each population was isolated by FACS (Fig. 3A), replated as a monolayer in cardiac-permissive conditions, and induced with doxycycline at day 4 to express *Gata5*. Abundant beating colonies were derived from the *Gata5*-expressing cells, including those that had been sorted as singly positive for KDR, and also, but less so, for those singly positive for PDGFRα (Fig. 3B). Although TNNT2$^+$ cells differentiated mostly from just the double-positive uninduced cells, immunohistochemistry documented abundant TNNT2$^+$ cells differentiated from the KDR$^+$/PDGFRα$^+$, KDR$^+$/PDGFRα$^-$ and KDR$^-$/PDGFRα$^+$ populations that were induced to express *Gata5* (Fig. 3C). In contrast, very few TNNT2$^+$ cells were generated by *Gata5* expression in the KDR$^-$/PDGFRα$^-$ population. These results suggest that *Gata5* can direct cardiac fate from non-cardiac mesoderm.

Previous studies have shown that GATA factors can expand cardiac fate in mesoderm at the expense of hematopoietic tissue (Schoenebeck et al., 2007; Simões et al., 2011), so we sought to determine whether this is the case in the EB system. However, regardless of whether *Gata5* expression was induced, we found that hematopoiesis was fully blocked in EBs cultured in the feeder-free, serum-free conditions (not shown). When the iGata5ES cells were reconditioned to grow in the presence of serum, the EBs could efficiently generate hematopoietic colonies. Expression of *Gata5* under these conditions did not inhibit hematopoiesis, and resulted in a modest increase in colony formation (supplementary material Fig. S6). However, under these conditions (with serum), the cardiogenic program was inefficiently activated by *Gata5*. Therefore, although *Gata5* does not directly repress hematopoiesis, how it affects a bi-potential hemato-cardiac progenitor can not be addressed in the ESC system, as the response to *Gata5* depends highly on the culture conditions, with serum supporting hematopoiesis, but suppressing cardiogenesis.

The effect of serum on *Gata5* cardiogenic activity led us to consider whether culture conditions affect *Gata4* activity, as all previous experiments showing a propensity for *Gata4* to direct endoderm (rather than cardiac) fate were performed in the presence of serum. To compare the activities directly, we conditioned the iGata4ES line for growth in feeder-free, serum-free conditions. We also generated analogous lines for *Gata6* by targeting a Flag-tagged *Gata6* cDNA into the doxycycline-inducible locus of AinV parental cells and conditioning these cells (iGata6ES) for culture in feeder-free, serum-free conditions. All three sets of cell lines were shown to differentiate as EBs with similar kinetics, with respect to the expression of KIT and KDR (not shown). For each line, GATA factor expression was induced with doxycycline at day 4 and activation of the cardiogenic program was compared by qPCR at day 5.

In contrast to serum-conditioned lines, each of these lines, including iGata4ES and iGata6ES, activated expression of cardiac regulatory genes *Tbx5*, *Mef2c*, *Nkx2.5* and *Isl1* (Fig. 4A), whereas mesoderm markers were unchanged (*Kdr*, *Pdgfra*) or suppressed (*Mesp1*, *Bry*). There were, however, some notable exceptions. For example, compared with *Gata4* or *Gata5*, *Gata6* poorly activated *Tbx5*, but much more effectively activated *Mef2c*. The differences might reflect early crosstalk among the genes themselves. For example, *Gata5* increases expression of *Gata4* 30-fold, whereas

Table 1. Action potential diversity in iGATA5-derived cardiomyocytes

Cell type (*n*)	RP	APA	APD_{50}	APD_{90}	APD_{90}/APD_{50}
Spontaneously beating cells					
Immature (9)	–65.7±1.2	85.7±2.7	216.8±36.2*	330.7±44.2	1.6±0.1
Nodal (5)	–51.2±4.7*	63.6±4.2*	90.4±13.6*	253.1±17.9	3.0±0.4*
Quiescent cells					
Atrial (19)	–63.4±1.3	96.6±2.7	61.2±6.9	195.5±16.6	3.5±0.3
Ventricular (7)	–71.1±2.0*	98.3±6.4	182.4±47.5*	264.0±64.5	1.5±0.1*

Comparison of action potential diversity in the four subtypes. RP, resting potential; APA, action potential amplitude; APD_{50}, action potential duration at 50% repolarization; APD_{90}, action potential duration at 90% repolarization. *P<0.05. The number of cells is indicated in parentheses.

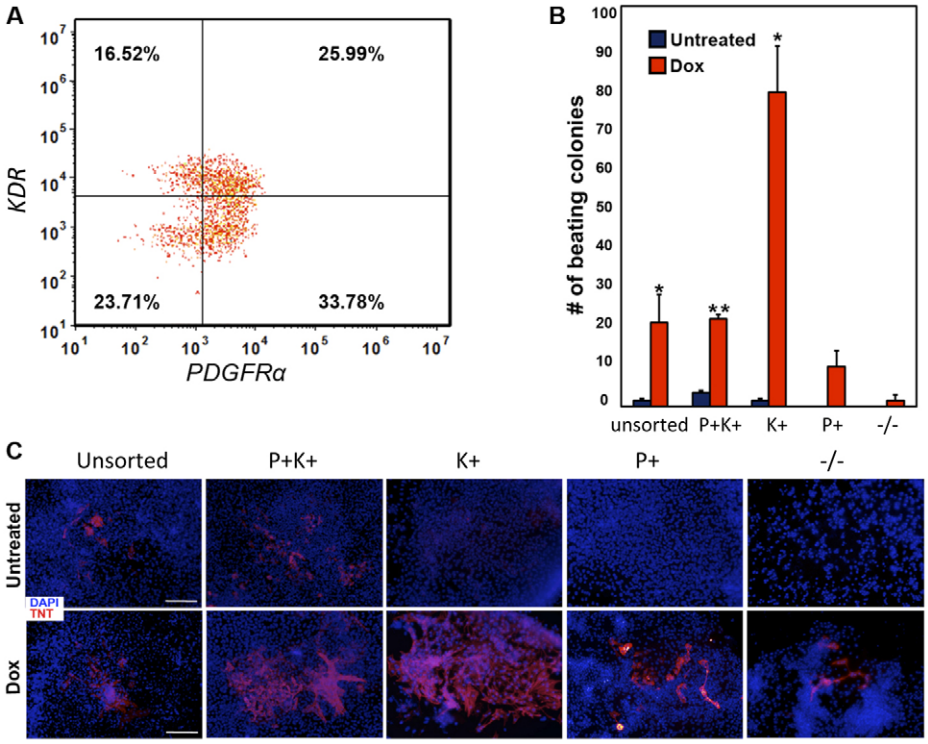

Fig. 3. *Gata5*-induced cardiac cells can be generated from KDR$^+$ mesoderm. (**A**) EB derivatives were sorted at day 4 to isolate by FACS the KDR$^-$/PDGFRα$^-$, KDR$^+$/ PDGFRα$^-$, KDR$^-$/PDGFRα$^+$ and KDR$^+$/PDGFRα$^+$ populations. A representative plot is shown. (**B**) Cells purified from 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.

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).

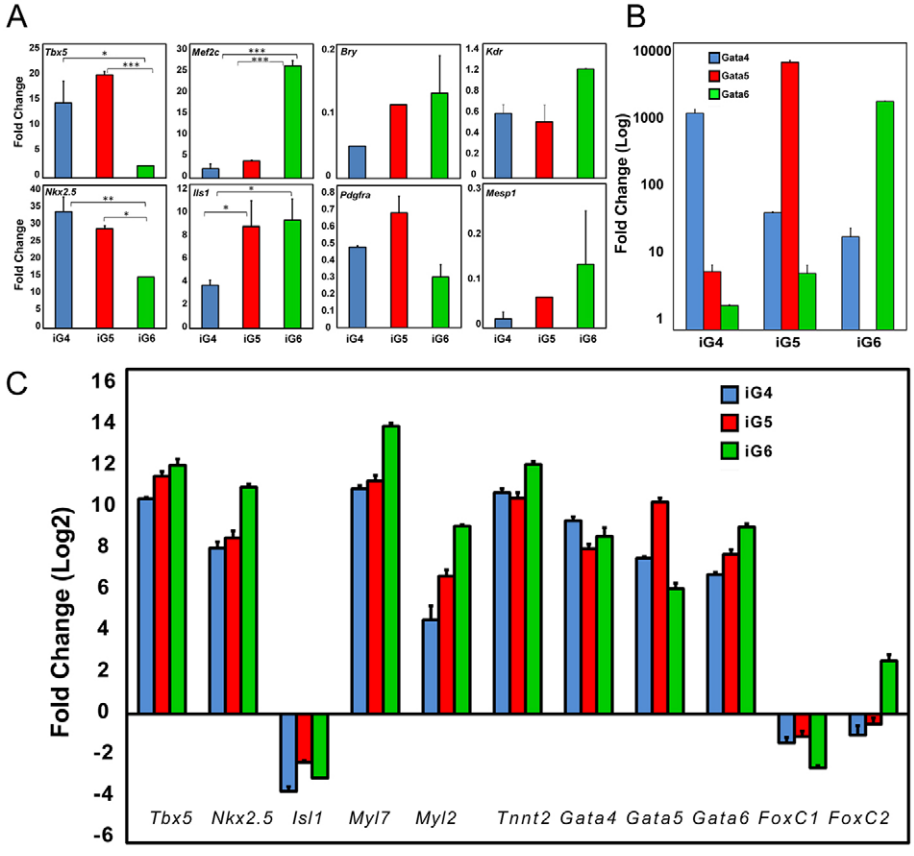

Fig. 4. Each of the GATA factors, under serum-free conditions, has capacity to induce cardiogenesis, but with distinctions. (**A**) Each line, iGata4ES (blue), iGata5ES (red) or iGata6ES (green) was induced at day 4 and RNA analyzed for the indicated cardiac genes (*Tbx5*, *Mef2c*, *Nkx2.5* or *Isl1*) 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 (Kume, 2009).

ESCs 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 *Mesp1* (David et al., 2008; Bondue and Blanpain, 2010). Consistent with its normal expression pattern in a wider mesodermal domain, *Mesp1* 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 *Mesp1* and upstream of *Tbx5*, *Nkx2.5* and *Mef2c*.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
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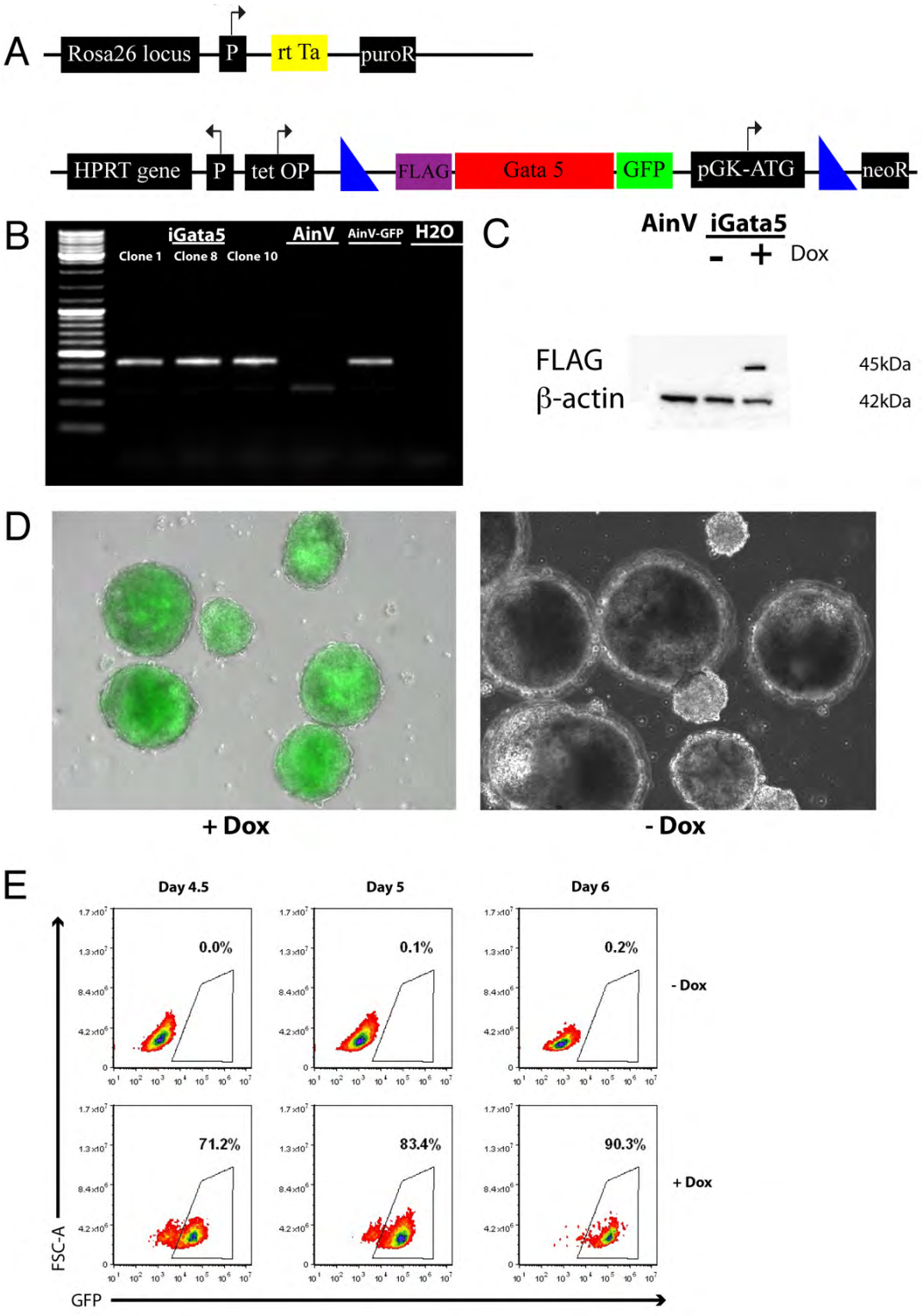

Fig. S1. Design and validation of iGata5ES cell lines. (**A**) In the parental Ainv ESC line, the reverse tet-transactivator protein (rtTa) is expressed from the Rosa26 locus. A loxP site (blue triangle) is engineered upstream of the HPRT promoter (P) and flanked by a tet operator (tet OP) site to generate a tetracycline-regulated promoter. It also includes a neomycin resistance gene that lacks a start codon (neoR). Cre-mediated recombination was used to target a flag-tagged *Gata5* cDNA, IRES:GFP cassette and phosphoglycerkinase promoter (pGK-ATG) into the loxP site, placing *Gata5* under conditional control. Both GATA5 and GFP are expressed, but only with addition of doxycycline. (**B**) Genomic PCR analysis (400 bp product) confirms single-copy site-specific integration of the cDNA-IRES:GFP cassette. Three separate clonal transgenic ESC lines are shown (clones 1, 8 and 10). The parental AinV18 line and H20 alone serve as negative controls, and a transgenic AinV clone with only a GFP cassette provides a positive control. (**C**) Western blot analysis of total cell extracts from parental AinV and uninduced (–) or doxycycline induced (+) iGata5ES-derived embryoid bodies. Protein detection was performed using a monoclonal anti-FLAG and anti-β-actin antibodies. (**D**) Fluorescence image (10×) of EBs derived from iGata5ES cells demonstrating strong GFP expression with doxycycline induction. (**E**) Flow cytometry analysis demonstrating GFP expression in EBs derived from iGata5ES at 12-48 hours after doxycycline induction.

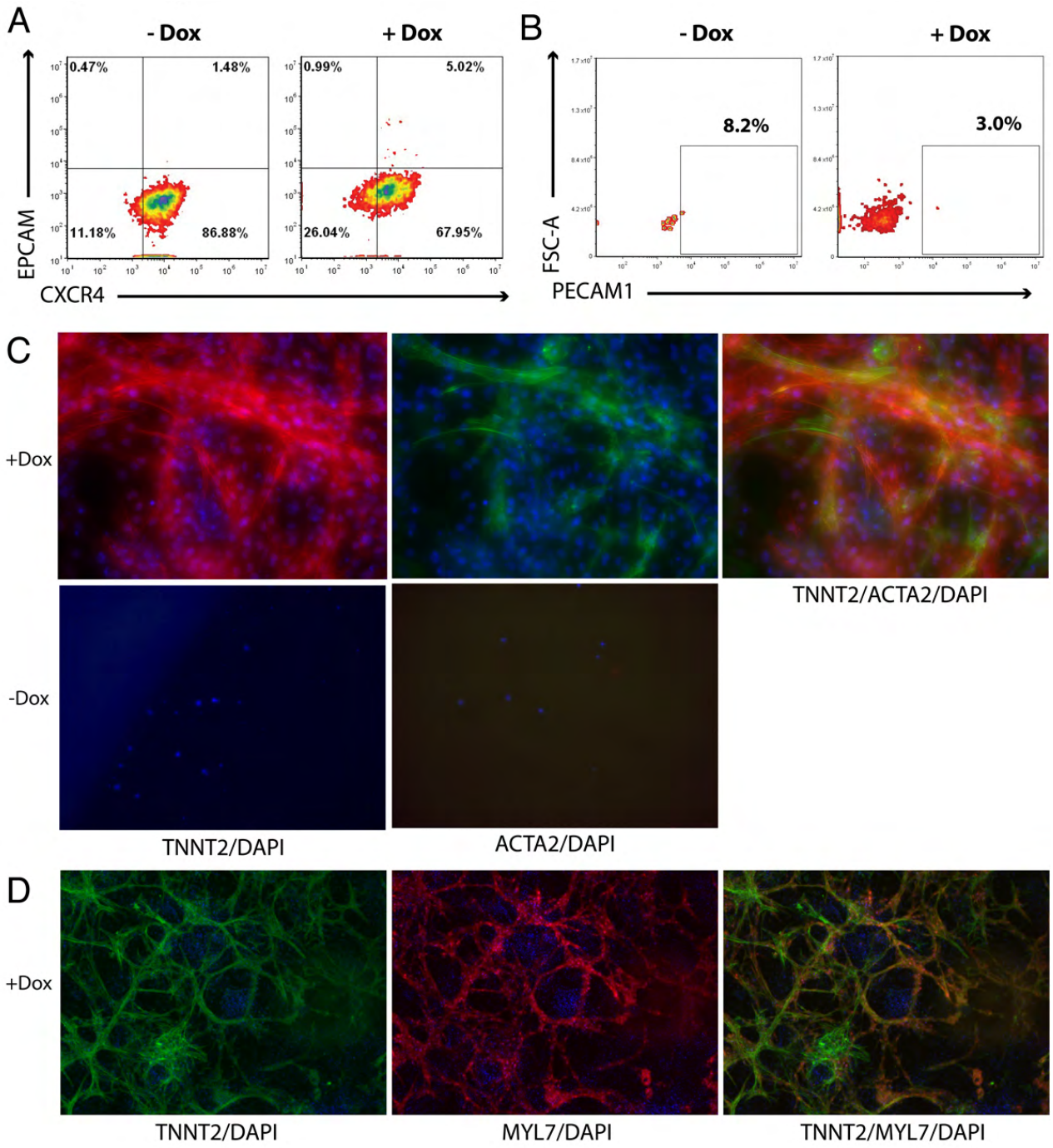

Fig. S2. *Gata5* expression directs the development of differentiated cardiac cells. (**A**) Flow cytometry analysis of day 6 EBs for EPCAM and CXCR4 with or without doxycycline induction of *Gata5*. (**B**) Flow cytometry analysis of day 12 EBs for PECAM1 with or without doxycycline induction of *Gata5*. (**C**) Induced (+Dox) and uninduced (–Dox) EBs were analyzed by immunohistochemistry for TNNT2 (left, red) and ACTA2 (middle, green) on day 12. Right panel is the merge. (**D**) Induced EBs were analyzed as in C for TNNT2 (left, green) and MYL7 (middle, red) on day 16. Right panel is the merge.

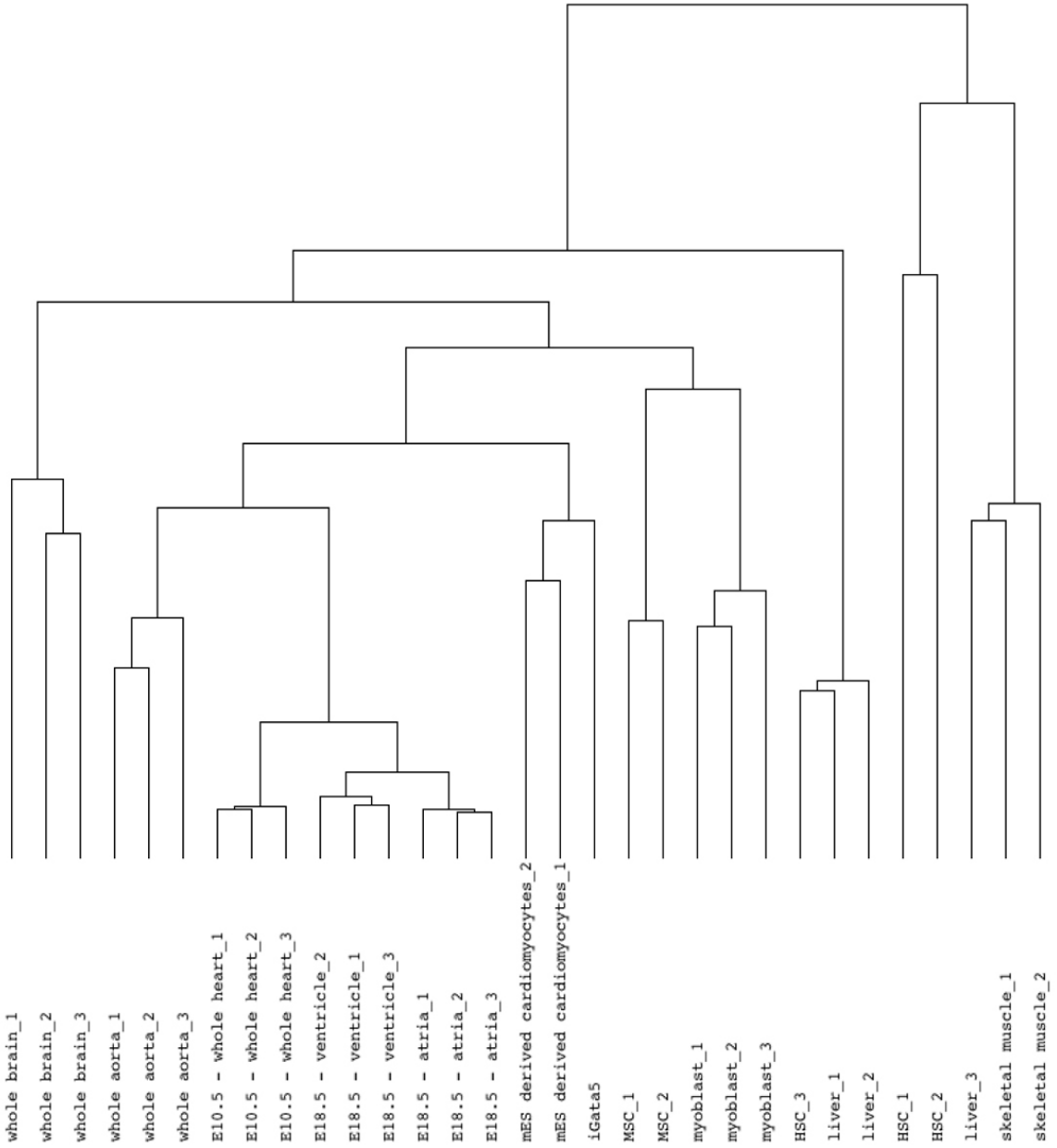

Fig. S3. RNA profiles of *Gata5*-induced EB derivatives cluster closely with mESC-derived cardiac precursors. ESC lines were induced with doxycycline at day 4, EB derivatives plated as a monolayer on fibronectin and RNA was isolated at day 16. After processing using a one-round in vitro transcription (IVT) system (MessageAmp Premier RNA Amplification Kit, Ambion/Applied Biosystems, Austin, TX), the cRNA was labeled with biotin, fragmented and hybridized to the Affymetrix Mouse Genome 430 Plus 2.0 GeneChip arrays (Santa Clara, CA). The array was scanned using GeneChip Scanner 3000 7G, and Affymetrix GeneChip Operating Software was used for image acquisition. The data normalization and statistical analysis were performed with Array Star 2 (DNASTAR, Madison, WI, USA). Shown is the dendrogram from an unsupervised hierarchical clustering of Gata5-induced EB derivatives in comparison with a variety of published microarray datasets (supplementary material Table S1).

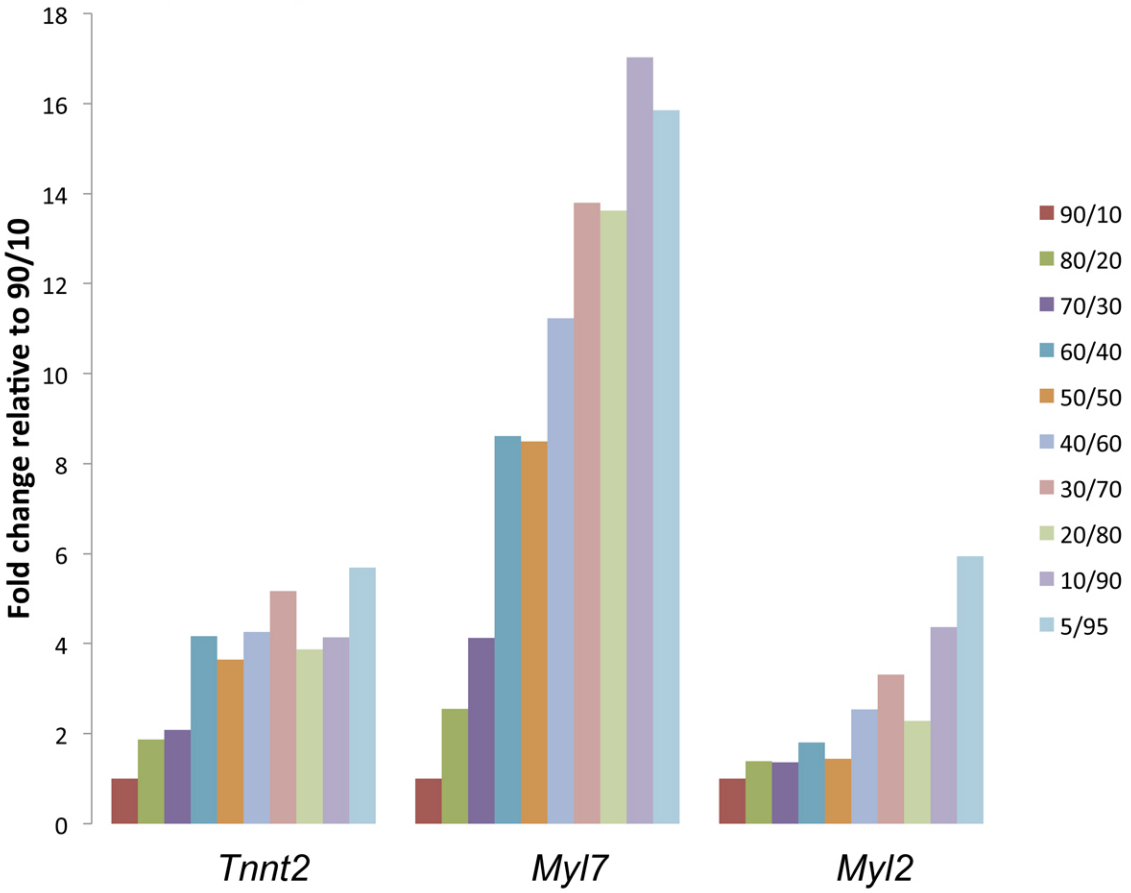

Fig. S4. Cardiac output is directly proportional to input of Gata5-expressing ESC derivatives. EB cultures were generated using mixed ratios of either parental AinV cells or the iGata5 doxycycline-inducible cells. EBs were induced at day 4 and harvested at day 6, and evaluated for expression of cardiac differentiation markers by qPCR, as in Fig. 1C. Shown are results from a representative experiment that was reproduced three times. For each gene product, transcript levels are normalized to the sample containing only 10% iGata5ES cells (far left red bar, 90/10). Moving to the right, each sample contained increasing percentage of iGata5ES cells, in the ratio of AinV to iGata5ES (e.g. 80/20). Although there is some sample variability, the increase in cardiac transcript levels is overall proportional to the input of iGata5ES cells.

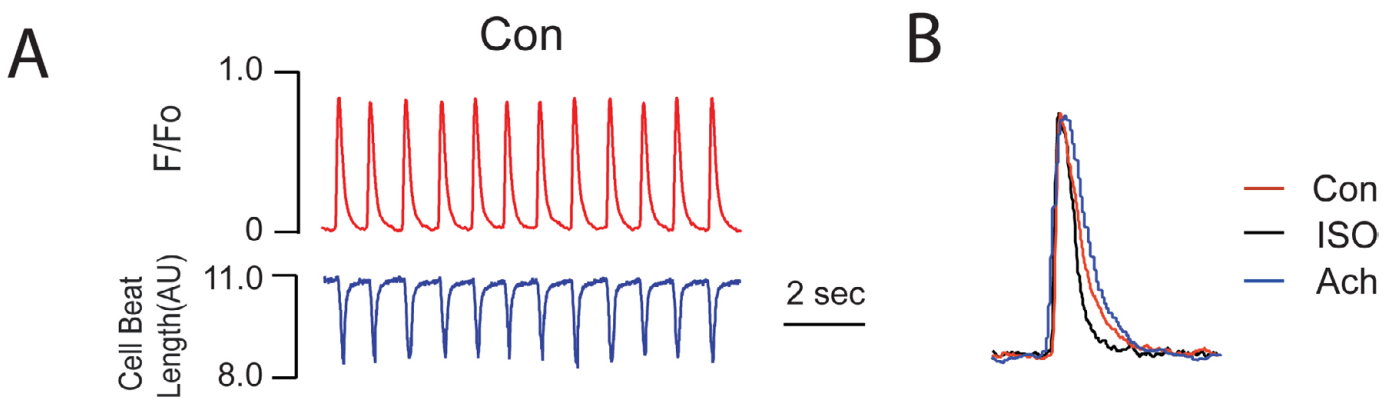

Fig. S5. *Gata5*-induced cardiac precursors demonstrate changes in Ca^{2+} oscillation kinetics. iGata5ES-derived cardiac precursors were dissociated with 0.25% trypsin-EDTA and plated onto laminin-coated coverslips at low density. Ca^{2+} fluorimetry was carried out using Fluo-4/AM. Cells were exposed to a dye-loading solution consisting of a standard Tyrodes solution. (**A**) A representative example of spontaneous Ca^{2+} oscillations and cell beat length changes in control iGata5ES-derived cardiac precursors. (**B**) Addition of 10 μM acetylcholine (Ach) or 1 μM isoproterenol (Iso) results in expected changes in Ca^{2+} oscillation kinetics as seen in superimposed tracings.

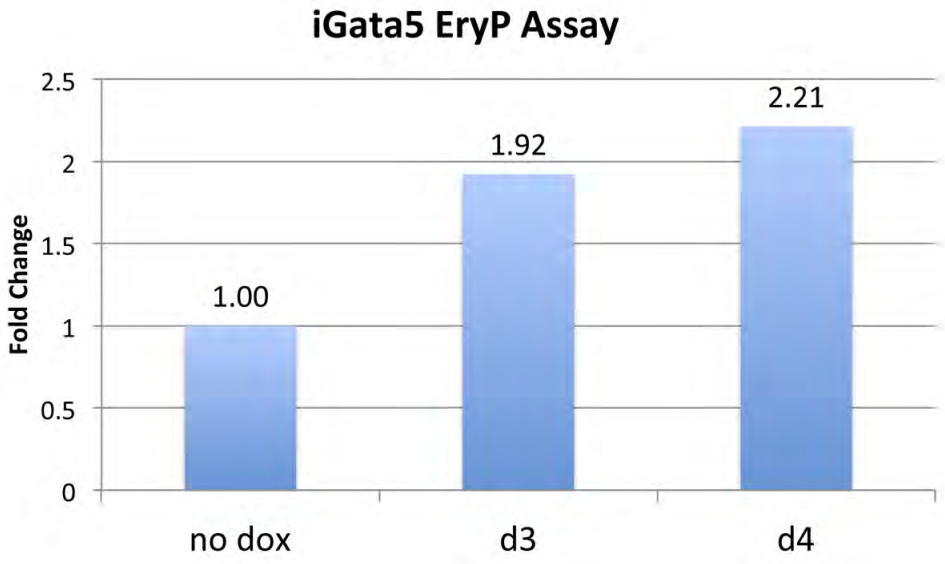

Fig. S6. Expression of Gata5 only modestly enhances hematopoiesis. In order to score hematopoietic progenitors, the iGata5ES cells were first re-conditioned to grow in the presence of serum on feeders, induced with doxycycline at day 3 or day 4. At day 6, an equivalent number of cells were plated with methycellulose in the presence of erythropoietin, and hematopoietic (primitive erythroid cell) colonies were counted 5 days later. Shown are representative results from a single experiment that was reproduced with similar results in three independent experiments.

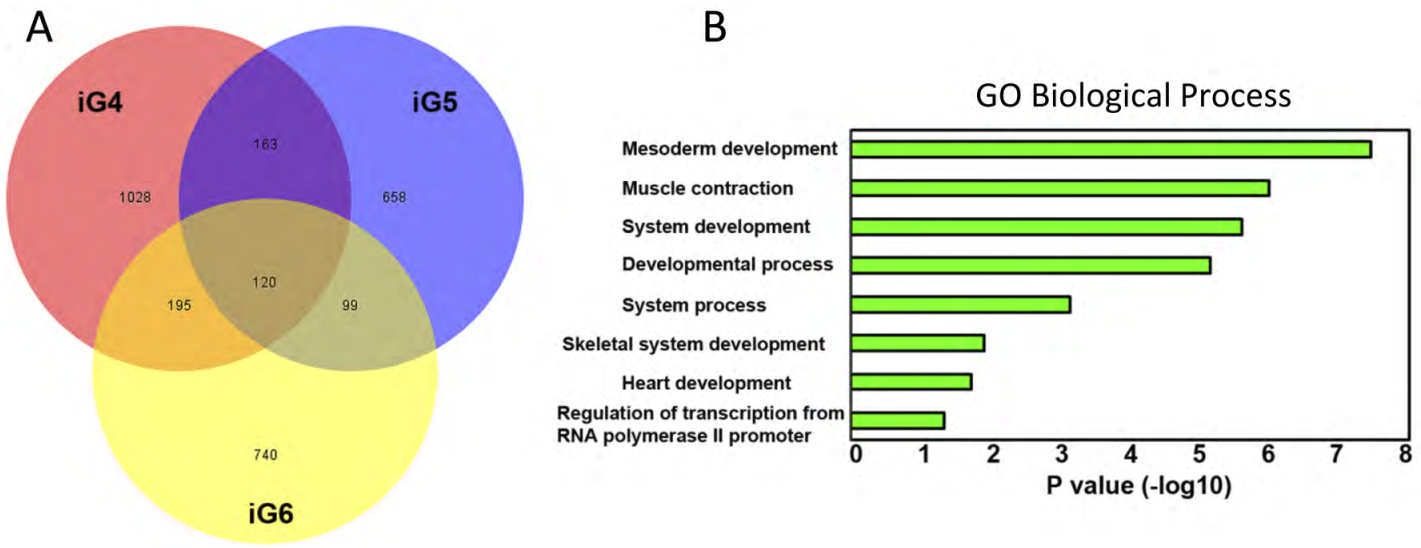

Fig. S7. Each of the GATA factors can robustly initiate cardiogenesis from ESCs. (**A**) In three independent experiments, EBs were induced with doxycycline at day 4 for 1 day and harvested for RNA profiling using Affymetrix microarrays, compared with uninduced controls. For each line, iGata4ES (iG4), iGata5ES (iG5) or iGata6ES (iG6), the gene sets activated or repressed at least fivefold compared with uninduced controls were compared (GenePattern). Venn diagrams indicate the overlap in these gene sets. (**B**) Gene ontology was analyzed for the triple-overlap set identified in A, comprising 120 genes that are highly associated with cardiac muscle developmental programs.

Movie 1. Monolayer of *Gata-5* induced EB derivatives displays extensive contractile activity. Real-time video of representative day 9 monolayer of EB derivatives on a fibronectin-coated well show extensive beating sheets of cardiomyocytes.

Table S1. List of microarray datasets used for hierarchical clustering

Label	GEO ID	Description
Whole brain 1,2,3	GSM 200695, 200696, 200697	C57BL/6 E9.5 whole brain
Whole aorta 1,2,3	GSM 238478, 238479, 238480	C57BL/6 2.5 to 4.5 month old whole aorta
E10.5 whole heart 1,2,3	GSM 25150, 25151, 25152	C57BL/6 E10.5 whole heart
E18.5 ventricle 1,2,3	GSM 25180, 25181, 25182	C57BL/6 E18.5 atrial chamber
E18.5 atria 1,2,3	GSM 25183, 25184, 25185	C57BL/6 E18.5 both ventricles
mES-derived cardiomyocytes 1,2	GSM 132680, 132681	mESC D3 transgenic line with neomycin resistance gene under the control of the alpha myosin heavy chain promoter (U71441)
MSC 1,2	GSM 476598, 476599	C57BL/6 bone marrow mesenchymal stem cells
HSC 1,2,3	GSM 291121, 291123	C57BL/6 8 week old hematopoietic stem cells
Myoblast 1,2,3	GSM 747404, 747405, 747406	C2C12 myoblast
Liver 1,2,3	GSM 300676, 300677, 300678	Mixed strain 28 day old liver
Skeletal muscle 1,2	GSM 385406, 385407	Wild type 7.5 month old gastrocnemius muscle

Table S2. List of qPCR primers

Gene	Forward (5′-3′)	Reverse (5′-3′)
Gapdh	CTAACATCAAATGGGGTGAGG	CGGAGATGATGACCCTTTTG
Nkx2.5	CATTTTACCCGGGAGCCTAC	CTTTGTCCAGCTCCACTGC
Tbx5	CAGGAGCACAGCCAAATTTAC	CCATGTACGGCTTCTTATAGGG
Gata4	TCTCACTATGGGCACAGCAG	GGGACAGCTTCAGAGCAGAC
FoxA2	GAGCCGTGAAGATGGAAGG	TCATGTTGCTCACGGAAGAG
Sox17	AGCAGAACCCAGATCTGCAC	GCTTCTCTGCCAAGGTCAAC
Nestin	AGGCGCTGGAACAGAGATT	GACATCTTGAGGTGTGCCAGT
Sox1	AACCCCAAGATGCACAACTC	TGTAATCCGGGTGTTCCTTC
Lmo2	ATCGAAAGGAAGAGCCTGGAC	GTCGATGGCTTTCAGGAAGTAG
Scl	AACAACAACCGGGTGAAGAG	CGCACTACTTTGGTGTGAGG
Cdh5 (*VE cad*)	ACCATCGCCAAAAGAGAGAC	TCTTGCCAGCAAACTCTCCT
Pecam1 (*Cd31*)	TGCACAGTGATGCTGAACAA	GCCTTCTGTCACCTCCTTTTT
Tnnt2 (*cTnt*)	CCTGCTGAGGCTGAACAGAT	CAGACATGCTCTCGGCTCTC
Myl7 (*Mlc2a*)	TTCTCATGACCCAGGCAGAC	CGTGGGTGATGATGTAGCAG
Myl2 (*Mlc2v*)	TGACCACACAAGCAGAGAGG	CCGTGGGTAATGATGTGGAC
Hcn4	GAGCCAGTACGCTCCAAACT	ACCTGAAGGAAGAAAGGAGCA
Gjc1 (*Conn45*)	TGGACTGCTGTAGTTACACTTTT	ACGAGAGGCACTTTTATTAAGTG

Table S3. List of antibodies

Primary	Secondary (flow cytometry)	Secondary (immunohistochemistry)
TNNT2 (CTNT) (Thermo, clone 13-11)	Anti-mouse IgG1 Alexa Fluor 647 (Invitrogen) Anti-mouse IgG Alexa Fluor 647 (Invitrogen) Anti-mouse IgG1 PE (eBioscience) Anti-mouse IgG1 FITC (Southern Biotech)	Anti-mouse IgG1 Alexa Fluor 568 (Invitrogen) Anti-mouse IgG1 FITC (Southern Biotech) Anti-mouse IgG Cy3 (Biomeda)
ACTA2 (SMA) (Abcam)	Anti-mouse IgG2a Alexa Fluor 488 (Invitrogen) Anti-mouse IgG Alexa Fluor 647 (Invitrogen)	Anti-mouse IgG2a Alexa Fluor 488 (Invitrogen)
PDGFRA (eBioscience)	*APC conjugated	n/a
KDR (FLK1) (eBioscience)	*PE conjugated	n/a
MYL7 (MLC2A) (Santa Cruz)	n/a	Anti-mouse IgG2b Alexa Fluor 568 (Invitrogen)