UCLA
UCLA Previously Published Works

Title

Inducible Cassette Exchange: A Rapid and Efficient System Enabling Conditional Gene Expression in Embryonic Stem and Primary Cells

Permalink

https://escholarship.org/uc/item/4z9529nq

Journal

Stem Cells, 29(10)

ISSN

1066-5099

Authors

Iacovino, Michelina
Bosnakovski, Darko
Fey, Holger
et al.

Publication Date

2011-10-01

DOI

10.1002/stem.715

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Nkx2-5 Represses *Gata1* Gene Expression and Modulates the Cellular Fate of Cardiac Progenitors During Embryogenesis

Arianna Caprioli, Naoko Koyano-Nakagawa, Michelina Iacovino, Xiaozhong Shi, Anwarul Ferdous, Richard P. Harvey, Eric N. Olson, Michael Kyba and Daniel J. Garry

Circulation 2011, 123:1633-1641: originally published online April 4, 2011
doi: 10.1161/CIRCULATIONAHA.110.008185
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
Copyright © 2011 American Heart Association. All rights reserved. Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/123/15/1633

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2011/03/31/CIRCULATIONAHA.110.008185.DC1.html

Subscriptions: Information about subscribing to Circulation is online at
http://circ.ahajournals.org//subscriptions/

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
http://www.lww.com/reprints

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

Nkx2-5 Represses *Gata1* Gene Expression and Modulates the Cellular Fate of Cardiac Progenitors During Embryogenesis

Arianna Caprioli, PhD*; Naoko Koyano-Nakagawa, PhD*; Michelina Iacovino, PhD*; Xiaozhong Shi, PhD; Anwarul Ferdous, PhD; Richard P. Harvey, PhD; Eric N. Olson, PhD; Michael Kyba, PhD; Daniel J. Garry, MD, PhD

Background—Recent studies suggest that the hematopoietic and cardiac lineages have close ontogenic origins, and that an early mesodermal cell population has the potential to differentiate into both lineages. Studies also suggest that specification of these lineages is inversely regulated. However, the transcriptional networks that govern the cell fate specification of these progenitors are incompletely defined.

Methods and Results—Here, we show that Nkx2-5 regulates the hematopoietic/erythroid fate of the mesoderm precursors early during cardiac morphogenesis. Using transgenic technologies to isolate Nkx2-5 expressing cells, we observed an induction of the erythroid molecular program, including *Gata1*, in the *Nkx2-5*–null embryos. We further observed that overexpression of Nkx2-5 with an Nkx2-5–inducible embryonic stem cell system significantly repressed *Gata1* gene expression and suppressed the hematopoietic/erythroid potential, but not the endothelial potential, of the embryonic stem cells. This suppression was cell-autonomous, and was partially rescued by overexpressing Gata1. In addition, we demonstrated that Nkx2-5 binds to the *Gata1* gene enhancer and represses the transcriptional activity of the *Gata1* gene.

Conclusions—Our results demonstrate that the hematopoietic/erythroid cell fate is suppressed via Nkx2-5 during mesodermal fate determination, and that the *Gata1* gene is one of the targets that are suppressed by Nkx2-5. **(*Circulation*. 2011; 123:1633-1641.)**

Key Words: developmental genes ■ gene expression regulation ■ Nkx2-5 protein, mouse

Previous studies support the hypothesis that multipotent progenitors give rise to diverse lineages in the developing embryo. Examples of multipotent progenitors include the mesoangioblast (which parents hematopoietic, smooth muscle, endothelial, and cardiomyocyte lineages[1]), hemangioblasts (a common progenitor of hematopoietic and endothelial lineages[2]), and multipotent cardiac progenitor cells, which have the potential to differentiate into all 3 major cell types of the heart, including cardiac myocytes, endothelial cells, and smooth muscle cells.[3] Although the multipotent cardiac progenitor cells express Nkx2-5, the transcriptional networks that govern the fate of these multipotent progenitors remain incompletely defined. Our previous analysis of the cardiac cell lineage demonstrated that the Nkx2-5–expressing progenitors transiently express hematopoietic markers, supporting the notion that there is a bipotential stage in early mesoderm during which cardiac and hematopoietic markers are coexpressed.[4]

Clinical Perspective on p 1641

Clinical Perspective on p 1641

Nkx2-5 is one of the earliest transcription factors expressed in multipotent cardiac progenitor cells during vertebrate heart development. Global elimination of *Nkx2-5* in mice results in severe growth retardation, perturbed cardiac morphogenesis, and embryonic lethality at approximately embryonic day (E) 9.5 to 10.0.[5,6] However, conditional elimination of *Nkx2-5* in cardiac myocytes results in viable neonates that have progressive cardiac dysfunction.[7] Although these studies highlight the significance of Nkx2-5 transcriptional activity during cardiac morphogenesis, they also support the notion that the embryonic lethality in mice with global disruption of *Nkx2-5* is due in part to perturbations of other lineages, because these null embryos have severe anemia, angiogenic defects, and the absence of endocardial cushion.[5,6] These results further indicate that the functional role of Nkx2-5 during embryogenesis is not restricted to promoting cardiac muscle development.

Received May 12, 2010; accepted February 15, 2011.

From the Center for Developmental Biology (A.C.), Department of Internal Medicine (A.F.), and Department of Molecular Biology (E.N.O.), University of Texas Southwestern Medical Center, Dallas; Lillehei Heart Institute, University of Minnesota, Minneapolis (N.K.-N., M.I., X.S., M.K., D.J.G.); and Victor Chang Cardiac Research Institute and Faculties of Medicine and Life Science, University of New South Wales, Sydney, Australia (R.P.H.).

*Drs Caprioli, Koyano-Nakagawa, and Iacovino contributed equally to this article.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.110.008185/DC1.

Correspondence to Naoko Koyano-Nakagawa, PhD, or Daniel J. Garry, MD, PhD, Lillehei Heart Institute, 420 Delaware St SE, MMC 508, Minneapolis, MN 55455. E-mail koyano@umn.edu or garry@umn.edu

© 2011 American Heart Association, Inc.

Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.110.008185**

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

Gata factors have been grouped on the basis of their expression and role during development. Gata1, Gata2, and Gata3 are involved in hematopoietic commitment, erythrocyte differentiation, and progenitor cell proliferation and T-cell differentiation, respectively, whereas Gata4, Gata5, and Gata6 play important roles during cardiac morphogenesis.[8] Gata1 is the first transcription factor shown to be important for the genesis of the erythroid lineage early during embryogenesis.[9] Targeted disruption or overexpression of Gata1 results in embryonic lethality caused by anemia, suggesting that Gata1 dosage levels are critically regulated during erythropoiesis. Previous studies have identified the essential regulatory elements for *Gata1* expression in erythroid lineages, and have established that *Gata1* gene expression is partly regulated by Gata1 itself.[9] Recent fate mapping studies in zebrafish defined that the hematopoietic and cardiac fates from lateral plate mesoderm are inversely regulated.[10] Although the previous study describes SCL and ER71 as transcription factors that promote the hematopoietic fate and repress the cardiac fate, the cardiac transcription factor that functions in a reciprocal fashion is yet to be defined.

In the present study, we define an Nkx2-5–mediated mechanism that coordinately regulates the cellular fate of mesoderm progenitors. Using genetic mouse models and molecular analyses of Nkx2-5–expressing cells, we observed an induction of the erythroid molecular program, including *Gata1*, in the progenitors isolated from the developing *Nkx2-5*–null hearts. We also demonstrated that overexpression of Nkx2-5, using an inducible embryonic stem cell/embryoid body (ES/EB) system, significantly repressed the hematopoietic/erythroid program, but not the endothelial program, in a cell-autonomous fashion. This repression was partially rescued by Gata1 overexpression. Using a reporter gene assay, we observed that Nkx2-5 represses the *Gata1* promoter activity by binding to the *Gata1* gene enhancer. Our studies support a model for Nkx2-5–dependent regulation of *Gata1* gene expression in the multipotent progenitors. This novel and previously undefined functional role for Nkx2-5 will complement and extend our understanding of the mechanisms by which cardiac lineages are determined in mesodermal precursors.

Methods

Transcriptional Assays

The 3.9-kb *Gata1* hematopoietic enhancer and minigene were previously described.[11] All transcriptional assays were performed in the K562 cell line. Specific conditions can be found in online-only Data Supplement.

Generation of Nkx2-5–Inducible Embryonic Stem Cell/Embryoid Body System

Generation and maintenance of inducible Nkx2-5 (iNkx2-5) cells, formation and differentiation of EBs were carried out as described.[12,13] Doxycycline (0.5 μg/mL) was added to induce Nkx2-5 expression for 24 hours at the start of day 3. EBs were washed and fed fresh medium on day 4 and further cultured for an additional 2 days to evaluate the effect of Nkx2-5 on hematopoietic commitment. To monitor the effect of Nkx2-5 on hematopoietic/erythroid differentiation, EBs were treated with doxycycline for 48 hours beginning on day 4 of EB formation. EBs were collected on day 6 and processed for isolation of RNA and fluorescence-activated cell sorter (FACS) analyses or for methylcellulose assays.

Statistical Analysis

Data represent the average of >3 replicates (replicate numbers are indicated in the text) and SD. Significance was tested by the Mann-Whiney *U* test for 2 groups and Kruskal-Wallis test, with the Dunn multiple-comparison test for >2 groups. For colony counts, normalizing transformation (square root) was done before statistical analysis. The above analyses were done with Prism 4.0 software (GraphPad Software). Quantitative reverse-transcription polymerase chain reaction (RT-PCR) data were analyzed by the RQ analysis algorithm (ABI). Error bars indicate the 99% confidence interval.

Results

Induction of the Erythroid Molecular Program in the *Nkx2-5*–Null Heart

We have previously generated a transgenic mouse model using an *Nkx2-5* cardiac-specific enhancer to direct enhanced yellow fluorescent protein (EYFP) reporter expression to the developing heart. This transgenic model recapitulates endogenous Nkx2-5 expression early during heart development.[4] Transcriptome analyses from EYFP-positive embryonic cells revealed that the cardiac transcriptional regulators are enriched in this population.[4] The analysis further revealed that the progenitors isolated from the E7.75 cardiac crescent have transient expression of genes that are essential for erythroid differentiation. These genes were downregulated at later stages of heart development (E9.5).[4] These data suggested that cardiac progenitors coexpress erythroid genes but that cardiac transcriptional regulators promote cardiac differentiation by negatively regulating the gene expression of the erythroid program.

To address the functional role of Nkx2-5 in this regulatory pathway, we examined the molecular signature of the EYFP-expressing cells in the presence or absence of Nkx2-5. We crossed *Nkx2-5*$^{+/-}$ female and *Nkx2-5-EYFP* transgenic *Nkx2-5*$^{+/-}$ male mice, and isolated EYFP-positive cells from the developing hearts of wild-type (*Nkx2-5*$^{+/+}$) or Nkx2-5–null (*Nkx2-5*$^{-/-}$) progeny. Persistent EYFP expression was observed in the heart field of both *Nkx2-5*$^{+/+}$ and *Nkx2-5*$^{-/-}$ embryos (Figure 1A).

The EYFP^{+} cells from individual wild-type and null littermate embryos at E8.0 were sorted with the use of flow cytometry and processed for gene expression analysis by quantitative RT-PCR (Figure 1B). We observed that *Nppa,* a direct downstream target of Nkx2-5,[5,14] was downregulated in the *Nkx2-5*$^{-/-}$ cardiac progenitor cells, whereas the hematopoietic markers *Gata1*, *Eklf*, and *Nfe2* were upregulated (Figure 1B). Thus, our analysis demonstrated increased expression of hematopoietic markers in Nkx2-5$^{-/-}$ progenitors.

Overexpression of Nkx2-5 Represses Erythroid Differentiation in ES Cells

We used the ES/EB system to enhance our understanding of the role of Nkx2-5 regulating hematopoietic fate, because it is a powerful model system for the study of cellular fate specification and lineage commitment.[15] Previous studies have demonstrated that the commitment of EBs to a hematopoietic fate occurs between days 2.75 and 3.75, whereas hematopoietic differentiation occurs between days 4 and 6.[16]

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

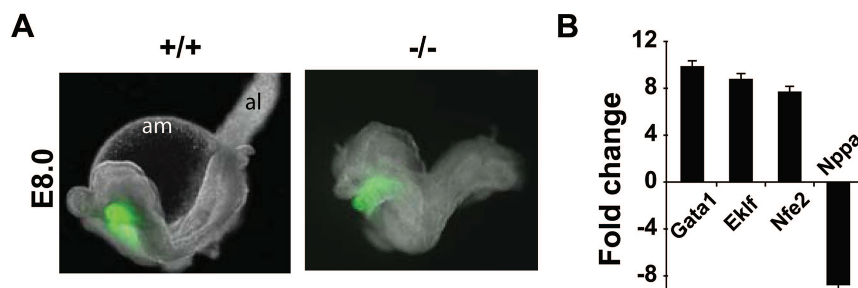


Figure 1. Upregulation of *Gata1* expression in *Nkx2-5*$^{-/-}$ progenitors. **A,** Age-matched *Nkx2-5-EYFP*$^{Tg/+}$*:Nkx2-5*$^{+/+}$ and *Nkx2-5-EYFP*$^{Tg/+}$*:Nkx2-5*$^{-/-}$ littermate embryos at embryonic day 8.0 (E8.0). Al indicates allantois; am, amnion. **B,** Quantitative reverse-transcription polymerase chain reaction shows that hematopoietic transcripts were significantly upregulated in the green fluorescent protein–positive (GFP$^+$) population in the *Nkx2-5*$^{-/-}$ embryos. Note the upregulation of *Gata1* and downregulation of *Nppa*, a known downstream target of Nkx2-5 in the absence of Nkx2-5. Bars indicate 99% confidence interval (n=3).

Moreover, our laboratory and others have demonstrated that Nkx2-5 expression is observed as early as in 3.5 days of EB differentiation, and is robustly expressed by day 5.[17–19] Because the onset of *Nkx2-5* expression (day 3.5) coincides with the divergence of hematopoietic and cardiac fate in the ES/EB system, we hypothesized that Nkx2-5 has a functional role in this process. To test this hypothesis, we generated an iNkx2-5 cell line, an ES cell line that expresses Nkx2-5 in response to doxycycline.[12] Nkx2-5 was induced for 24 hours from day 3 to 4 EBs to examine the effect on hematopoietic commitment or for 48 hours from day 4 to 6 EBs to evaluate the effect on hematopoietic differentiation, and the respective EBs were collected on day 6 (Figure 2A).

Using quantitative RT-PCR, we observed that genes involved in erythroid differentiation are downregulated in doxycycline-induced EBs with both treatment schedules (Figure 2B). In contrast, a downstream target gene of Nkx2-5 in cardiac development, *Nppa*, was significantly upregulated (Figure 2B). Therefore, Nkx2-5 does not act as a nonspecific repressor of gene expression, but specifically inhibits the erythroid program in doxycycline-induced EBs. The reciprocal response of these genes to overexpression and loss of function of Nkx2-5 supports the notion that Nkx2-5 inhibits the erythroid program while promoting cardiac development (Figure 1B and 2B).

Using FACS analysis, we then examined the cell surface markers of iNkx2-5 cells. Control and doxycycline-induced EBs were dissociated at day 6 of differentiation and analyzed for c-kit and CD41 (hematopoietic progenitor markers) versus c-kit and CD45 (more committed hematopoietic cell markers) expression. When Nkx2-5 was induced from day 3 to day 4, we identified no hematopoietic progenitors (c-kit$^+$/CD41$^+$) or mature hematopoietic cells (c-kit$^+$/CD45$^+$; data not shown). When *Nkx2-5* was overexpressed between days 4 and 6 of differentiation, both early and late hematopoietic progenitors (c-kit$^+$/CD41$^+$ and c-kit$^+$/CD45$^+$) were detectable, but significantly reduced in the presence of Nkx2-5 (Figure 2C and 2D).

To further evaluate whether Nkx2-5 may also play a role in hematopoietic/erythroid differentiation of ES cells, we used colony-forming cell assays. Untreated day 6 EBs were trypsinized, and 5×10^4 cells were plated on methylcellulose plates for hematopoietic colony formation in the presence or absence of doxycycline. Colonies were quantified after 6 days of culture. Few or no colonies were formed from 2 independent clones of iNkx2-5 cells when doxycycline was added to the methylcellulose, whereas erythroid, granulocyte-macrophage, and mixed colonies were detected when cultured in the absence of doxycycline (Figure 2E and 2F). To determine whether Nkx2-5 overexpression specifically affected the erythroid program or has a broader role in hematopoiesis, day 4 EBs were induced for 48 hours (with variable concentrations of doxycycline) and then plated on methylcellulose in the absence of doxycycline (Figure 2G). Induction of Nkx2-5 expression in EBs at this earlier time of hematopoietic development resulted in a preferential repression of erythroid colony formation in a dose-dependent fashion (Figure 2G). The reduction of erythroid colonies was readily observed at the lowest dose of doxycycline (0.1 μg/mL) examined, whereas formation of other colonies was less affected. Collectively, our data support the hypothesis that Nkx2-5 either directly or indirectly represses the expression of genes that are essential for erythroid specification and differentiation during development.

Overexpression of Nkx2-5 Does Not Repress Endothelial Differentiation in Embryonic Stem Cells

Because previous studies have suggested that hematopoietic and endothelial cells as well as cardiomyocyte and endothelial cells are derived from common progenitors,[16,17,20] we examined whether overexpression of Nkx2-5 also repressed the endothelial specification/differentiation of EBs. Day 4 EBs were induced with doxycycline or left uninduced for 48 hours, dissociated on day 6, and analyzed with FACS. Cells were doubly stained with combinations of antibodies against Flk-1, VE-cadherin, and CD31 (platelet endothelial cell adhesion molecule [PECAM]) to identify endothelial lineages. We observed that the induction of Nkx2-5 expression did not repress the endothelial program (Figure 3A and 3B), in contrast to the strong repression of hematopoietic/erythroid potential (Figure 2). These results indicated that overexpression of Nkx2-5 specifically repressed hematopoietic/erythroid potential, but not the endothelial/vascular potential, of the differentiating EBs.

To further confirm these results, we used morphological techniques of serial sections. An antibody against CD31/PECAM as well as hematoxylin and eosin staining were used

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

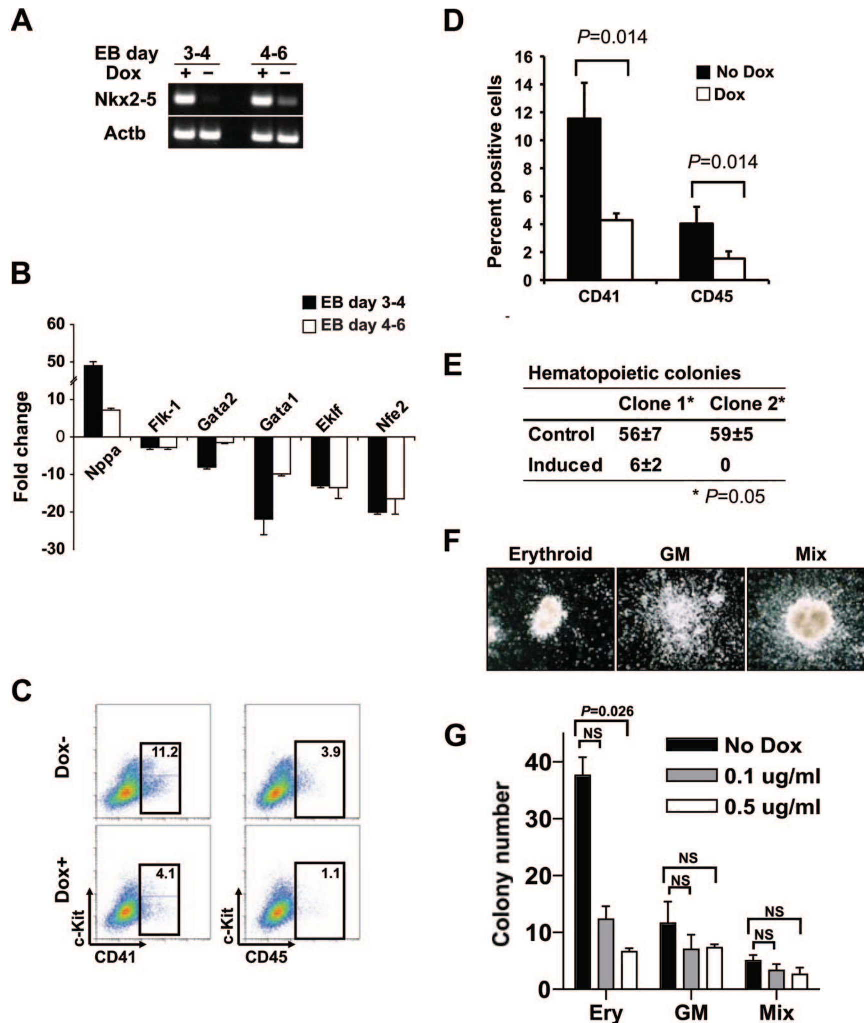


Figure 2. Overexpression of Nkx2-5 results in repression of the hematopoietic/erythroid lineages. **A,** Induction of the *Nkx2-5* transcript in inducible Nkx2-5 (iNkx2-5) cells was verified by reverse-transcription polymerase chain reaction (RT-PCR). β-Actin (Actb) is a loading control. **B,** Quantitative RT-PCR analysis of doxycycline-treated and untreated embryoid bodies (EBs; n=6) revealed that overexpression of Nkx2-5 significantly suppressed erythroid genes, including *Gata1*. In contrast, an Nkx2-5 downstream target gene, *Nppa*, was significantly upregulated. Bars represent 99% confidence interval (n=3). **C,** Fluorescence-activated cell sorter (FACS) analysis of cell surface markers of differentiating EB cells. EBs were treated (Dox+) or left untreated (Dox−) from day 4 to 6 and analyzed for hematopoietic markers on day 6. For early and late hematopoietic markers, combinations of c-kit/CD41 and c-kit/CD45, respectively, were used. The experiment was repeated 5 times, and representative data are shown. **D,** Quantification of the FACS data in **C** (analyzed by Mann-Whitney *U* test; n=5). **E** and **F,** Induction of Nkx2-5 expression suppresses hematopoietic/erythroid colony formation. **E,** Day 6 EBs from 2 different clones of iNkx2-5 cells were isolated and plated in complete methylcellulose in the absence (control) or presence (induced) of doxycycline (analyzed by Mann-Whitney *U* test after normalizing transformation; n=3). Note that little or no colony formation was observed in the presence of doxycycline, whereas erythroid, granulocyte-macrophage (GM), and mixed colonies were detected when EBs were cultured without doxycycline. **G,** Induction of Nkx2-5 expression from day 4 to 6 with lower concentrations of doxycycline preferentially repressed erythroid colony formation. Day 4 EBs with or without 48-hour induction with the specified concentration of doxycycline were dissociated, and equal numbers of cells were plated in methylcellulose without doxycycline. Note the significant repression of erythroid (ery) colony formation compared with the GM and mixed colonies (analyzed by Mann-Whitney *U* test after normalizing transformation; n=3).

to identify vessel-like structures (VLSs) and blood cells, respectively. As shown in Figure 3C, CD31/PECAM-positive VLSs were detected in both induced and noninduced EBs, but VLSs containing blood-like cells were detected almost exclusively in the noninduced EBs. Quantification of VLSs from both doxycycline-treated and untreated EBs revealed that only 7% of the VLSs contained blood-like cells in the Nkx2-5–induced EBs, whereas ≈60% of the VLSs had blood components in the noninduced EBs (Figure 3D). These data further confirmed that the overexpression of Nkx2-5 negatively regulated the hematopoietic/erythroid program without affecting the endothelial differentiation of progenitor cells.

Nkx2-5 Represses Hematopoietic Differentiation of Embryonic Stem Cells Cell-Autonomously

We examined whether repression of hematopoietic differentiation by Nkx2-5 is mediated by secreted factors or is a cell-autonomous effect. We mixed iNkx2-5 cells with E14–green fluorescent protein (GFP) cells, an ES cell line that constitutively expressed the GFP protein,[21] and differentiated

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

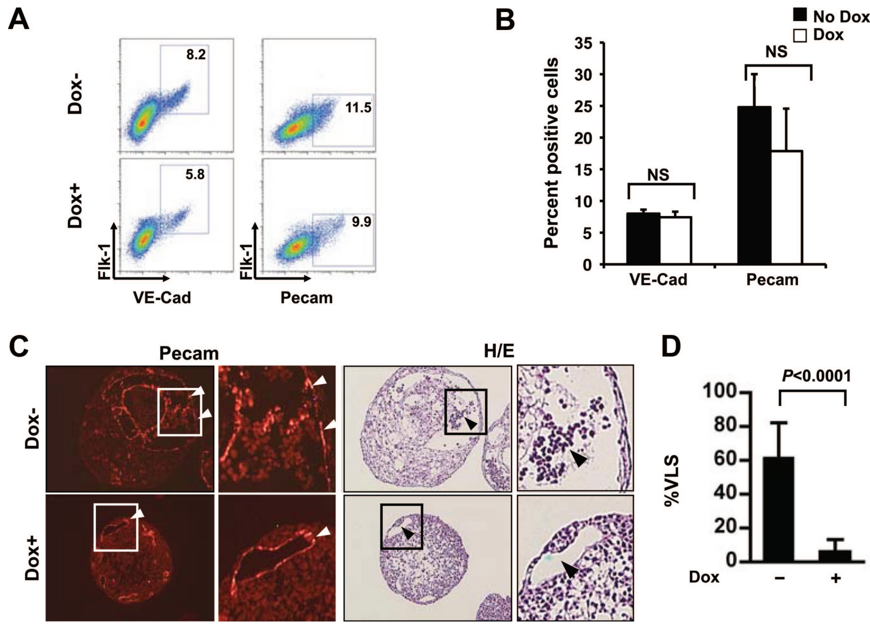


Figure 3. Overexpression of Nkx2-5 does not affect endothelial differentiation of embryonic stem cell/embryoid body (ES/EB). **A** and **B,** Cells from day 6 EBs treated (Dox+) or untreated (Dox−) with doxycycline for 48 hours were analyzed for expression of indicated markers using fluorescence-activated cell sorter (FACS). Note that overexpression of Nkx2-5 did not affect the endothelial progenitors in the differentiating EBs. These FACS data are quantified in **B** (analyzed by 1-tailed Mann-Whitney *U* test; n=3). **C** and **D,** Adjacent sections of treated and untreated day 6 EBs were stained with anti-CD31/platelet endothelial cell adhesion molecule (PECAM) antibody (left) or hematoxylin and eosin (H/E; right) to analyze the formation of vessel-like structures (VLS; white arrowheads) and blood cells (black arrowheads). Boxed areas are enlarged. Three hundred randomly selected VLS from 20 EBs were scored for the presence of blood cells in **D** (analyzed by 1-tailed Mann-Whitney *U* test; n=33). Bars show SD.

EBs in the presence or absence of doxycycline (Figure 4A). We analyzed the EBs on day 6 of induction. Green fluorescent protein-positive and -negative cells were isolated and analyzed for CD41 expression (as a measure of hematopoiesis) with FACS. When iNkx2-5 cells and E14-GFP cells were mixed at a 50:50 ratio, the hematopoietic potential of iNkx2-5 cells (GFP-negative population) was suppressed after treatment with doxycycline (Figure 4B; compare a and c). In contrast, the hematopoietic potential of E14-GFP cells was not significantly altered in the same culture (Figure 4B; compare b and d). To address whether a putative soluble factor is limiting in the culture, we mixed the cells at a 75:25 ratio, but the hematopoietic potential of E14-GFP cells still was not affected (Figure 4C). Thus, these data demonstrated that Nkx2-5 repressed hematopoietic differentiation of iNkx2-5 cells in a cell-autonomous fashion.

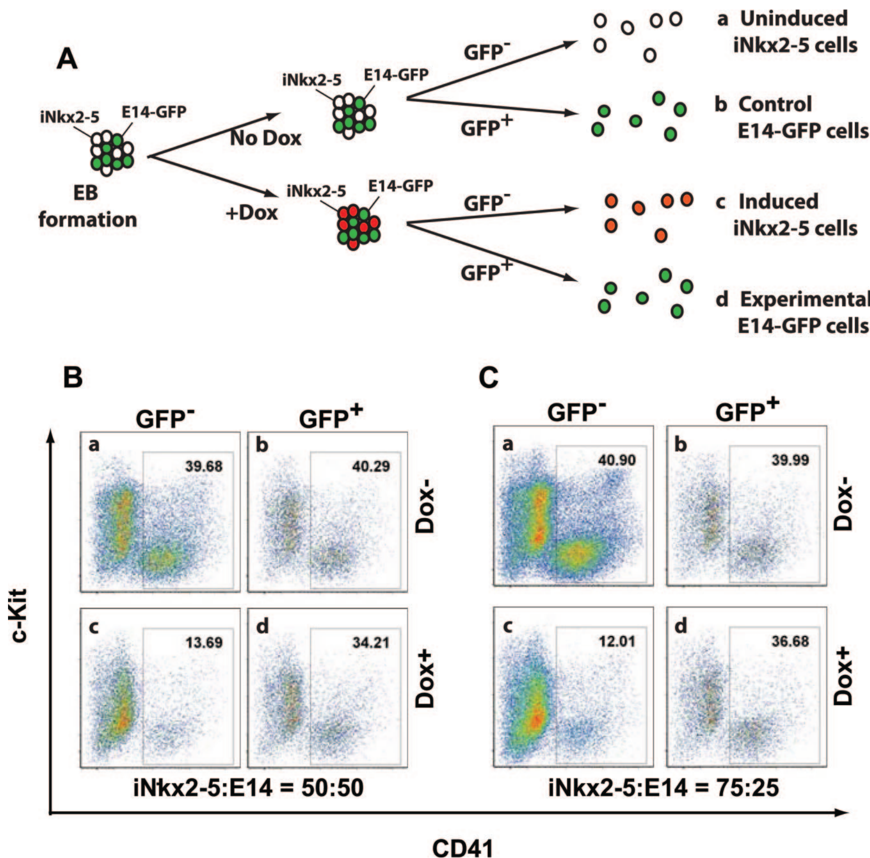


Figure 4. Nkx2-5 suppresses hematopoiesis in a cell-autonomous fashion. **A,** Inducible Nkx2-5 (iNkx2-5) cells were mixed with wild-type E14–green fluorescent protein (GFP) cells and induced to form embryoid bodies (EBs). Cells were either treated with doxycycline from day 2 to 4 or left untreated. On day 6, cells were dissociated and analyzed by fluorescence-activated cell sorter (FACS). **B** and **C,** Representative FACS profiles from experiments in which iNkx2-5 cells and E14-GFP cells were mixed at a 50:50 (**B**) or 75:25 (**C**) ratio. Note that in both conditions, the $CD41^+$ cells originated from iNkx2-5 cells ($CD41^+$, GFP^- population) were reduced on Nkx2-5 induction (compare a and c), whereas those that originated from E14-GFP cells ($CD41^+$, GFP^+ population) remained unchanged (compare b and d).

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

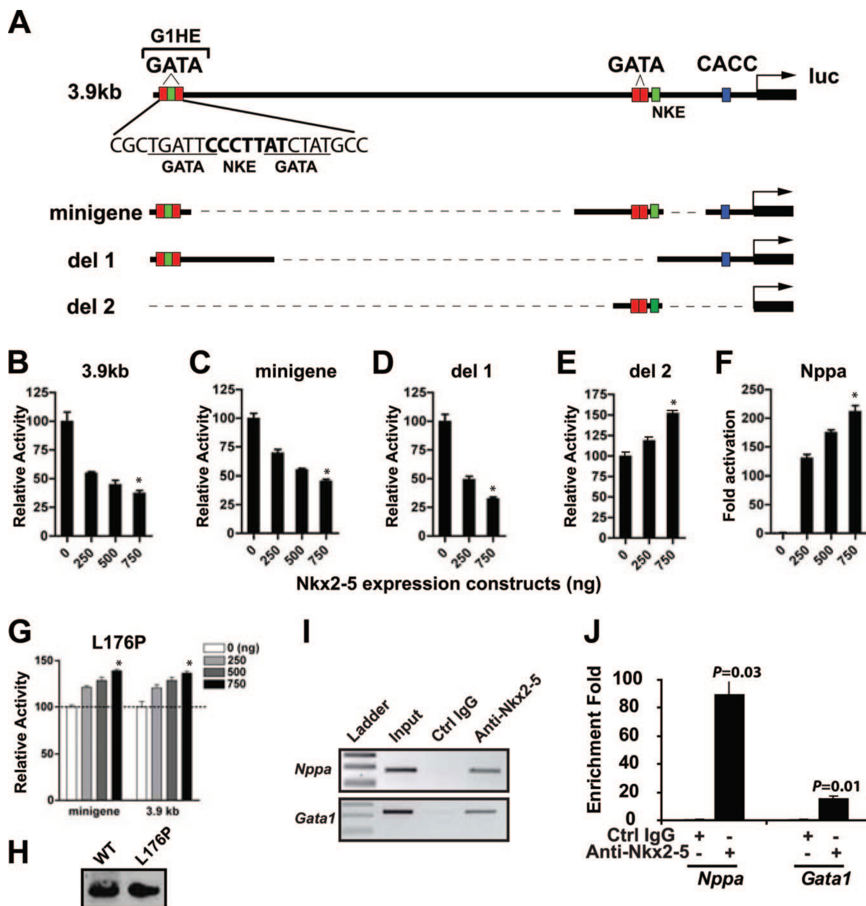


Figure 5. Nkx2-5 represses *Gata1* promoter activity. **A,** Schematic of the 3.9 kb upstream region of the *Gata1* gene and reporter plasmids used in the transcriptional assays. Two GATA binding sequences (underlined) and an Nkx2-5 consensus sequence (bold) within the G1HE are indicated. **B** through **G,** Transcriptional assay in K562 cells reveals a dose-dependent repression of luciferase activity by Nkx2-5 using the 3.9-kb erythroid enhancer (**B**) or the minigene (**C**). Deletion (del) 2 (**E**) but not deletion 1 (**D**) abolished the response to Nkx2-5. **F,** Nppa, a downstream target of Nkx2-5, was activated under the same condition. **G,** The L176P mutation of Nkx2-5 in the homeodomain impairs its activity to repress Gata1 transcription. **B** through **G** were analyzed by the Kruskal-Wallis test ($n=3$). *$P<0.05$ vs the sample without the Nkx2-5 expression vector (0 ng). Bars without an asterisk were not significant. **H,** Western blot analysis from the transfected samples (500-ng vector) shows that wild-type protein and the L175P mutant are expressed at equivalent levels. **I** and **J,** Chromatin was isolated from embryoid bodies (EBs) of inducible Nkx2-5 cells induced for 24 hours with doxycycline and analyzed by chromatin immunoprecipitation assay. **I,** Nkx2-5 antibody, but not control IgG, precipitated genomic fragments containing the *Gata1* and the *Nppa* promoter region. **J,** Quantification of the band intensity by quantitative polymerase chain reaction (analyzed by Wilcoxon signed rank test; $n=4$).

Nkx2-5 Represses *Gata1* Transcriptional Activity

Gata1 is an essential transcription factor for the genesis of the erythroid lineage early during embryogenesis.[22] Because *Gata1* and its downstream targets (eg, *Eklf1* and *Nfe2*)[23]were dysregulated with loss of function or overexpression of Nkx2-5, we hypothesized that the *Gata1* gene is regulated by Nkx2-5. Previous studies established that the 3.9-kb upstream sequence is essential for *Gata1* expression in both primitive and definitive erythroid cells (Figure 5A).[11,24,25] Four elements, including the *Gata-1* hematopoietic enhancer (G1HE) region, 2 proximal GATA-binding sites, and the CACCC motif, are necessary for *Gata1* expression, and the minigene construct (Figure 5A) that contains these 4 elements recapitulates *Gata1* gene expression in primitive erythroid cells.[11]

When the 3.9-kb reporter plasmid was cotransfected with increasing amounts of the Nkx2-5 expression vector, the luciferase activity was repressed in a dose-dependent fashion (Figure 5B). The minigene construct responded similarly to Nkx2-5 (Figure 5C). Two sequences show homology to the Nkx binding site (NKE) within the minigene construct (Figure 5A). Deletion of the proximal double GATA site and NKE (del1) reduced the baseline activity of the promoter but did not change its response to Nkx2-5 (Figure I in the online-only Data Supplement and Figure 5D). In contrast, when G1HE was deleted (del2), the repression by Nkx2-5 was abolished (Figure 5E). Similarly, the point mutation of the distal NKE (mut1), but not the proximal NKE (mut2), significantly reduced the response to Nkx2-5 (Figure II in the online-only Data Supplement). Thus, our results demonstrated that the 233-bp fragment harboring G1HE and NKE therein mediates the repressive activity of Nkx2-5. The repressive activity of Nkx2-5 was not a general inhibition of transcription because the promoter of *Nppa*, a known target of Nkx2-5, was activated >100-fold in the same system (Figure 5F).

Next, we determined whether Nkx2-5 directly binds to the *Gata1* gene. We generated a DNA binding mutant of Nkx2-5 (leucine 176 to proline; L176P) that functions as a dominant negative inhibitor of Nkx2-5.[26] Transient transfection assay demonstrated that the L176P DNA binding mutant did not inhibit the activity of the 3.9-kb upstream region or the Gata1 minigene, although the protein was expressed at levels similar to the wild-type (Figure 5G and 5H). Next, we examined whether Nkx2-5 binds to the G1HE region using chromatin immunoprecipitation assay. A specific band corresponding to the G1HE and *Nppa* regulatory region was amplified from immunoprecipitates using the Nkx2-5 antibody but not the control immunoglobulin (Figure 5I and 5J). Furthermore, electrophoretic mobility shift assay using nuclear extracts prepared from iNkx2-5 cells induced for 6 hours (to ensure that there is no secondarily induced genes) identified a specific shift that appears only in the induced extract (Figure III in the online-only Data Supplement). Collectively, our results support the conclusion that Nkx2-5 binds to the distal NKE and represses *Gata1* gene transcription.

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

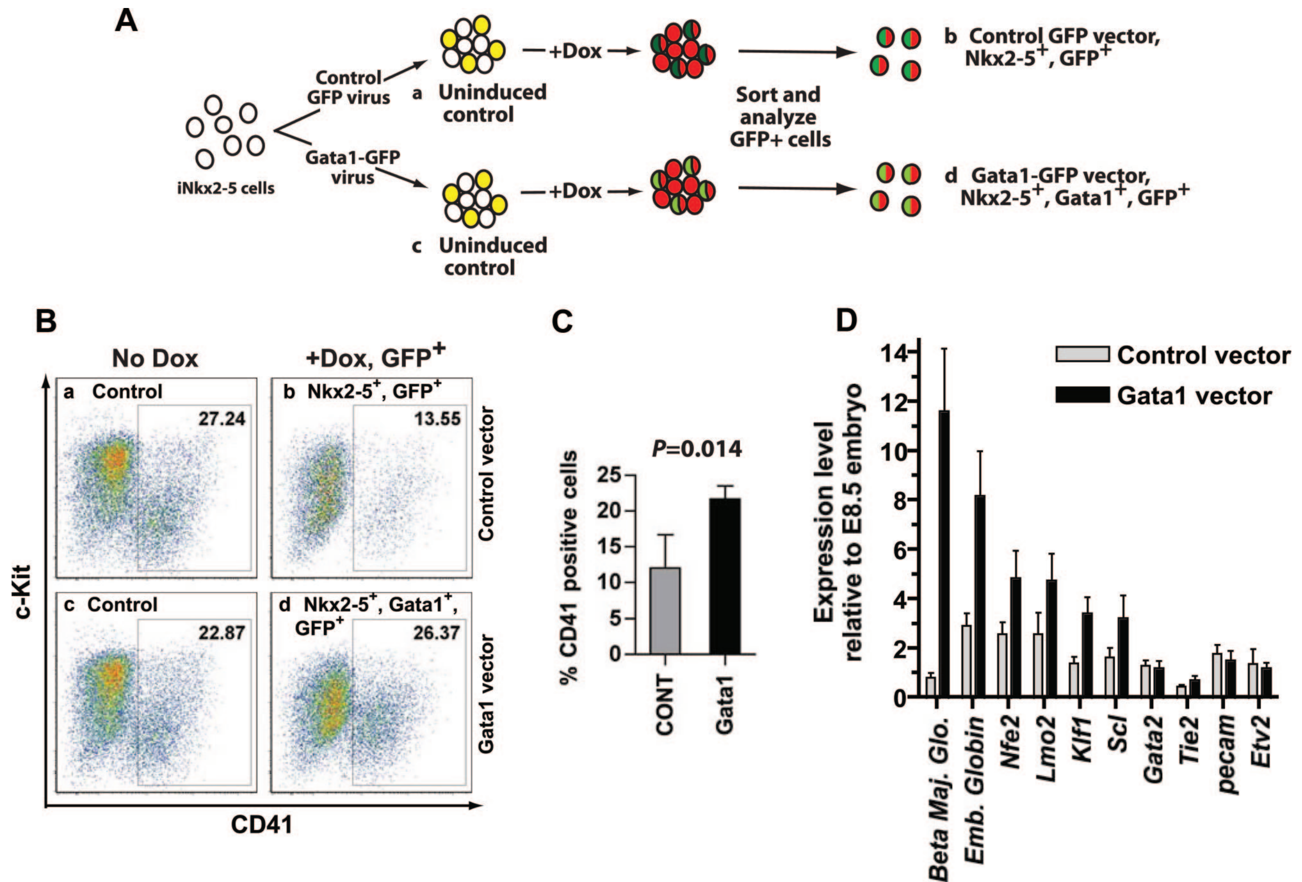


Figure 6. Gata1 partially rescues repression of hematopoiesis by Nkx2-5. **A,** Inducible Nkx2-5 (iNkx2-5) cells were infected with a lentiviral vector that expresses Internal Ribosome Entry Site (IRES)–green fluorescent protein (GFP) or a vector that expresses Gata1-IRES-GFP in response to doxycycline. Infected cells were enriched by sorting for GFP-positive cells and induced to form embryoid bodies (EBs). After inducing from day 4 to 6, cells were dissociated and analyzed for CD41 expression. The GFP-positive cells in the control group express Nkx2-5 and GFP (group b), whereas those in the experimental group express Nkx2-5, Gata1, and GFP (group d). **B,** Representative fluorescence-activated cell sorter (FACS) profile of groups a through d. **C,** Quantification of the FACS analyses. Mean number and SD from 4 independent experiments are shown. Data were analyzed by 1-tailed Mann-Whitney *U* test (n=4). **D,** Cells from groups b and d were collected, and gene expression was analyzed by quantitative reverse-transcription polymerase chain reaction. Note the upregulation of hematopoietic/erythroid markers by Gata1 overexpression. Bars indicate 99% confidence interval (n=3).

Repression of Hematopoietic Genes by Nkx2-5 Is Rescued by Overexpression of Gata1

To determine whether *Gata1* is a direct downstream target of Nkx2-5, we performed a rescue experiment (Figure 6A). We used an inducible lentiviral vector to express GFP alone (control) or the Gata1 and GFP proteins in response to doxycycline. Thus, in this system, expression of both *Nkx2-5* and *Gata1* was regulated by the tetracycline-responsive promoter. iNkx2-5 cells were infected, expanded, sorted for GFP-positive cells and were differentiated to form EBs. Embryoid bodies were treated with 1.0 μg/mL doxycycline from day 4 to 6 of induction and analyzed on day 6 for expression of CD41. In this experimental paradigm, all cells treated with doxycycline expressed Nkx2-5 (Figure 6A, groups b and d [red]). Sorting and analyzing GFP-positive cells allowed us to examine only the infected cells with the control GFP virus (group b [dark green]) or the Gata1-GFP virus (group d [light green]). Fluorescence-activated cell sorter analysis demonstrated that 22% to 28% of uninduced groups (groups a and c) were positive for CD41, whereas group b, which expressed Nkx2-5 and GFP, contained on average 12.0% of CD41-positive cells (Figure 6C). Group d, which expressed Nkx2-5, GFP, and Gata1, contained on average 21.7% of CD41-positive cells, indicating that Gata1 partially rescued the Nkx2-5–mediated repression (as measured by CD41 expression; Figure 6C). Congruent with the FACS profile, the gene expression profile of the sorted cells demonstrated a 15-fold increase in the *Gata1* transcript and between a 2- and 12-fold increase in erythroid-specific genes such as *beta major globin*, *embryonic globin*, *Nfe2*, *Lmo2*, *Klf1*, and *Scl* (Figure 6D). Thus, our data demonstrated that overexpression of Gata1 at least partially rescued the inhibitory effect of Nkx2-5 on hematopoiesis.

Discussion

Multipotent cardiac progenitors express Nkx2-5 and generate diverse lineages that contribute to heart formation during embryogenesis. Although intense interest has focused on the genesis of the cardiac progenitors, the molecular networks that govern cellular fate specification remain incompletely defined. In the present study, we used an array of technologies to define the functional role of Nkx2-5 and fate specifi-

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

cation. Our studies have advanced this field through several significant findings. First, using a genetic marking method, we observed that genes involved in erythroid differentiation are upregulated in the *Nkx2-5*$^{-/-}$ progenitors. This finding led us to hypothesize that an undescribed function of Nkx2-5 is to suppress noncardiac lineages during embryogenesis. This hypothesis was tested with the ES/EB system, which mirrors embryonic development and has been used as a model system to analyze the fate of cell lineages. We showed that overexpression of Nkx2-5 represses hematopoietic/erythroid differentiation in ES/EBs as assessed by 4 independent measures: gene expression pattern, expression of cell surface markers, morphology, and colony-forming activity. We further demonstrated that repression of hematopoietic/erythroid differentiation is a cell-autonomous effect. Interestingly, our cellular and immunohistochemical analyses revealed that overexpression of Nkx2-5 specifically and significantly repressed the hematopoietic/erythroid program, but not the endothelial potential, of the differentiating EBs. From this observation, we hypothesize that Nkx2-5 does not function at the level of the hemangioblast, the putative common precursor for hematopoietic and endothelial cells. Rather, our studies suggest that Nkx2-5 may function distinctly to maintain 1 lineage (endothelial or cardiomyocyte) by repressing gene expression associated with other (ie, hematopoietic) lineages.

Whether a common progenitor for cardiac and hematopoietic lineages exists has been controversial. Although analyses based on colony-forming assays show that hematopoietic and cardiac lineages arise from distinct mesodermal progenitors,[17,27] several lines of evidence suggest that there is a stage during development when mesodermal cells have multilineage potential and can generate blood or cardiac cells, depending on the environment.[4,10,28] For example, chick tissues from the posterior primitive streak, an area outside the heart field, can be induced to differentiate into cardiac myocytes by coculturing with anterior endoderm tissue. This takes place at the expense of the blood lineage.[28] In the zebrafish *cloche* mutant, specification of endothelial and hematopoietic lineages is impaired in the anterior lateral plate mesoderm, and in turn, the cardiac lineage is expanded.[10,29] Similar findings have been obtained in the ER71$^{-/-}$ embryos, in which blood and vessel development was severely repressed but cardiac markers were overexpressed.[30] Furthermore, in mouse embryos, it has been shown that the epiblast tissue maintains the hematopoietic potential during gastrulation, even after the period of hematopoietic differentiation is over and cardiac specification is initiated.[31] These observations support the notion that there is a biopotential or multipotential stage in early mesoderm and that activation of the cardiac program antagonizes the hematopoietic program. In the present study, we propose that Nkx2-5 has a dual role of promoting a cardiac fate and repressing hematopoietic fate in multipotent mesodermal progenitors. There is an apparent paradox with the previous report that hematopoiesis is impaired in Nkx2-5 mutant embryos.[5] We speculate that Nkx2-5 function is required in multiple stages of mesoderm development, and that the apparent lack of yolk sac hematopoiesis reflects an earlier function of Nkx2-5.[5,32] With the ability to identify progenitor cell populations earlier during embryogenesis (E6.5 to E7.5), future studies will be aimed at defining the roles of Nkx2-5 in multiple stages of mesoderm development.

Using transcriptional assays, we showed that one of the targets of Nkx2-5 is the *Gata1* gene, an essential transcription factor in erythroid differentiation. *Gata1* expression is strongly suppressed in Nkx2-5–overexpressing ES cells, and overexpression of Gata1 rescued the inhibitory effect of Nkx2-5 on hematopoietic development. The region in the *Gata1* minigene that is responsible for Nkx2-5–mediated repression coincided with G1HE. Although the absolute requirement of G1HE in erythroid transcription remains controversial,[33] G1HE maps with an erythroid-specific *DNase*I-hypersensitive site and is required for expression of the *Gata1* gene in primitive and definitive erythroid cells.[9] Mutation of the Gata binding site within G1HE severely impairs the promoter activity. Our chromatin immunoprecipitation assay and electrophoretic mobility shift assay demonstrated that Nkx2-5 binds to the G1HE region of the *Gata1* gene. This region contained an evolutionarily conserved sequence that shows homology to the Nkx2-5 recognition sequence (Figure 5A). Further work is warranted to investigate the molecular nature of Nkx2-5–mediated transcriptional repression. It is interesting to note that Nkx2-5 not only activates transcription, but also can inhibit transcription.[34] Our findings suggest that hematopoietic genes are additional targets of the suppressive activity of Nkx2-5.

Conclusions

Our data have uncovered a novel biological function for Nkx2-5 that represses hematopoietic/erythroid but not the vascular potential of the mesodermal progenitors. These studies further enhance our understanding of the regulatory mechanisms that govern cardiac morphogenesis, and may contribute to regenerative pathways involving cardiac progenitor cell populations after injury of the adult heart.

Acknowledgments

We thank Alicia Wallis, Camille Walter, and Danielle Rux for technical assistance. We thank Masayuki Yamamoto for the *Gata1* promoter construct and Benoit Bruneau for the Nppa/atrial natriuretic factor reporter plasmid.

Sources of Funding

We received funding support from the National Institutes of Health (U01 HL100407, R01 HL085729, R01 HL081186) and the American Heart Association (Jon Holden DeHaan Foundation, 0970499N).

Disclosures

Dr Garry is an Established Investigator of the American Heart Association.

References

1. Cossu G, Bianco P. Mesoangioblasts: vascular progenitors for extravascular mesodermal tissues. *Curr Opin Genet Dev*. 2003;13:537–542.
2. Mikkola HK, Orkin SH. The search for the hemangioblast. *J Hematother Stem Cell Res*. 2002;11:9–17.
3. Garry DJ, Olson EN. A common progenitor at the heart of development. *Cell*. 2006;127:1101–1104.

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

4. Masino AM, Gallardo TD, Wilcox CA, Olson EN, Williams RS, Garry DJ. Transcriptional regulation of cardiac progenitor cell populations. *Circ Res*. 2004;95:389–397.
5. Tanaka M, Chen Z, Bartunkova S, Yamasaki N, Izumo S. The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development*. 1999;126:1269–1280.
6. Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, Harvey RP. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev*. 1995;9: 1654–1666.
7. Pashmforoush M, Lu JT, Chen H, Amand TS, Kondo R, Pradervand S, Evans SM, Clark B, Feramisco JR, Giles W, Ho SY, Benson DW, Silberbach M, Shou W, Chien KR. Nkx2-5 pathways and congenital heart disease; loss of ventricular myocyte lineage specification leads to progressive cardiomyopathy and complete heart block. *Cell*. 2004;117: 373–386.
8. Patient RK, McGhee JD. The GATA family (vertebrates and invertebrates). *Curr Opin Genet Dev*. 2002;12:416–422.
9. Kaneko H, Shimizu R, Yamamoto M. GATA factor switching during erythroid differentiation. *Curr Opin Hematol*. 2010;17:163–168.
10. Schoenebeck JJ, Keegan BR, Yelon D. Vessel and blood specification override cardiac potential in anterior mesoderm. *Dev Cell*. 2007;13: 254–267.
11. Ohneda K, Shimizu R, Nishimura S, Muraosa Y, Takahashi S, Engel JD, Yamamoto M. A minigene containing four discrete cis elements recapitulates GATA-1 gene expression in vivo. *Genes Cells*. 2002;7:1243–1254.
12. Iacovino M, Hernandez C, Xu Z, Bajwa G, Prather M, Kyba M. A conserved role for Hox paralog group 4 in regulation of hematopoietic progenitors. *Stem Cells Dev*. 2009;18:783–792.
13. Dang SM, Kyba M, Perlingeiro R, Daley GQ, Zandstra PW. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnol Bioeng*. 2002;78:442–453.
14. Durocher D, Chen CY, Ardati A, Schwartz RJ, Nemer M. The atrial natriuretic factor promoter is a downstream target for Nkx-2.5 in the myocardium. *Mol Cell Biol*. 1996;16:4648–4655.
15. Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev*. 2005;19:1129–1155.
16. Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. *Development*. 1998; 125:725–732.
17. Kattman SJ, Huber TL, Keller GM. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell*. 2006;11:723–732.
18. Martin CM, Meeson AP, Robertson SM, Hawke TJ, Richardson JA, Bates S, Goetsch SC, Gallardo TD, Garry DJ. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. *Dev Biol*. 2004;265:262–275.
19. Wu SM, Fujiwara Y, Cibulsky SM, Clapham DE, Lien CL, Schultheiss TM, Orkin SH. Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell*. 2006;127: 1137–1150.
20. Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development*. 1998;125:1747–1757.
21. Ismailoglu I, Yeamans G, Daley GQ, Perlingeiro RC, Kyba M. Mesodermal patterning activity of SCL. *Exp Hematol*. 2008;36:1593–1603.
22. Harigae H. GATA transcription factors and hematological diseases. *Tohoku J Exp Med*. 2006;210:1–9.
23. Layon ME, Ackley CJ, West RJ, Lowrey CH. Expression of GATA-1 in a non-hematopoietic cell line induces beta-globin locus control region chromatin structure remodeling and an erythroid pattern of gene expression. *J Mol Biol*. 2007;366:737–744.
24. Onodera K, Takahashi S, Nishimura S, Ohta J, Motohashi H, Yomogida K, Hayashi N, Engel JD, Yamamoto M. GATA-1 transcription is controlled by distinct regulatory mechanisms during primitive and definitive erythropoiesis. *Proc Natl Acad Sci U S A*. 1997;94:4487–4492.
25. Vyas P, McDevitt MA, Cantor AB, Katz SG, Fujiwara Y, Orkin SH. Different sequence requirements for expression in erythroid and megakaryocytic cells within a regulatory element upstream of the GATA-1 gene. *Development*. 1999;126:2799–2811.
26. Toko H, Zhu W, Takimoto E, Shiojima I, Hiroi Y, Zou Y, Oka T, Akazawa H, Mizukami M, Sakamoto M, Terasaki F, Kitaura Y, Takano H, Nagai T, Nagai R, Komuro I. Csx/Nkx2-5 is required for homeostasis and survival of cardiac myocytes in the adult heart. *J Biol Chem*. 2002; 277:24735–24743.
27. Kouskoff V, Lacaud G, Schwantz S, Fehling HJ, Keller G. Sequential development of hematopoietic and cardiac mesoderm during embryonic stem cell differentiation. *Proc Natl Acad Sci U S A*. 2005;102: 13170–13175.
28. Schultheiss TM, Xydas S, Lassar AB. Induction of avian cardiac myogenesis by anterior endoderm. *Development*. 1995;121:4203–4214.
29. Gering M, Yamada Y, Rabbitts TH, Patient RK. Lmo2 and Scl/Tal1 convert non-axial mesoderm into haemangioblasts which differentiate into endothelial cells in the absence of Gata1. *Development*. 2003;130: 6187–6199.
30. Lee D, Park C, Lee H, Lugus JJ, Kim SH, Arentson E, Chung YS, Gomez G, Kyba M, Lin S, Janknecht R, Lim DS, Choi K. ER71 acts downstream of BMP, Notch, and Wnt signaling in blood and vessel progenitor specification. *Cell Stem Cell*. 2008;2:497–507.
31. Kanatsu M, Nishikawa SI. In vitro analysis of epiblast tissue potency for hematopoietic cell differentiation. *Development*. 1996;122:823–830.
32. Stanley EG, Biben C, Elefanty A, Barnett L, Koentgen F, Robb L, Harvey RP. Efficient Cre-mediated deletion in cardiac progenitor cells conferred by a 3′UTR-ires-Cre allele of the homeobox gene Nkx2-5. *Int J Dev Biol*. 2002;46:431–439.
33. McDevitt MA, Shivdasani RA, Fujiwara Y, Yang H, Orkin SH. A "knockdown" mutation created by cis-element gene targeting reveals the dependence of erythroid cell maturation on the level of transcription factor GATA-1. *Proc Natl Acad Sci U S A*. 1997;94:6781–6785.
34. Nimura K, Ura K, Shiratori H, Ikawa M, Okabe M, Schwartz RJ, Kaneda Y. A histone H3 lysine 36 trimethyltransferase links Nkx2-5 to Wolf-Hirschhorn syndrome. *Nature*. 2009;460:287–291.

CLINICAL PERSPECTIVE

The heart is the first organ to develop during embryogenesis. Genetic perturbations during development result in congenital heart disease, which is associated with considerable morbidity and mortality. To enhance our understanding of congenital heart disease, intense interest has focused on the definition of genetic networks that govern stem cells to form lineages such as the heart, vasculature, or blood. In the present study, we examined the hypothesis that Nkx2-5, in a context-dependent manner, regulates the formation of the heart and blood lineages. These studies advance our understanding of the molecular regulation of cardiac stem cells and have implications for congenital heart disease and cardiac regeneration.

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

Online Data Supplements

Supplemental Methods

Chromatin Immunoprecipitation (ChIP)

The ChIP assay was performed with the protocol described previously [1]. Briefly, iNkx2-5 cells were induced to form EBs, treated with 1 μg/ml doxycycline from day 4 to day 6, collected, washed with PBS, and treated with 0.25% trypsin for 5 minutes. The EB cells were triturated in culture medium, washed with PBS, fixed with 1% formaldehyde for 10 minutes at room temperature, and then resuspended in SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH8.1). The chromatin DNA was sheared 4 x 10 seconds at 20% amplitude with Sonic Dismembrator Model 500 to the fragment of 200–1000 base pairs. The DNA-protein complex was immunoprecipitated with the Nkx2-5 antibody (Santa Cruz, H-114). Quantitative PCR was performed to detect the upstream fragment in *ANF* or *Gata1* genes with primer pairs: TCAGCTTTTGTCCGTCACTG (ANF-forward) and ACAAGCTTTGCCGAACTGAT (ANF-reverse) or GCATGGGTCTCAAATGGAAG (Gata1-forward) and AGGGTGCCTCTAAGGACAGG (Gata1-reverse), respectively.

EMSA

Undifferentiated iNKX2-5 cells were treated or left untreated with 1 μg/ml doxycycline for 6 hours to induce Nkx2-5 expression and collected. At this time point, majority of the induced protein is Nkx2-5 and secondary targets of Nkx2-5 are not induced. Nuclear extracts were prepared with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce) and EMSA was done using Gel Shift assay Core Kit (Promega). Oligonucleoties used (upper strand) were

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

CCGCTGATTCCCTTATCTATGCCTTCC (wild type) and CCGCTGATTCC*G*TATCTATGCCTTCC (mutated). For competition experiments twenty fold excess of oligonucleotides were added prior to probe addition.

Generation of the iNkx2-5 ES cell line and differentiation by EB formation

Generation and maintenance of iNkx2-5 cells, an ES cell line in which myc epitope-tagged Nkx2-5 can be induced to overexpress by doxycycline[2], and formation and differentiation of embryoid bodies (EBs)[3] were carried out as described.

To evaluate the effect of Nkx2-5 on hematopoietic commitment, doxycycline (0.5 µg/ml) was added to EBs for 24 hours from Day 3 to Day 4, and then washed and cultured for another two days. To examine the effect of Nkx2-5 on hematopoietic/erythroid differentiation, EBs were treated with doxycycline for 48 hours beginning on Day 4 of EB formation. EBs were collected on Day 6 and processed for quantitative qRT-PCR and FACS analysis or for methylcellulose assay. FACS analysis was performed using the FACS Aria after staining the cells with the following fluorophore-conjugated antibodies: CD41, CD45, c-kit, Flk-1 and VE-cadherin (Pharmingen). Antibodies were used at a concentration of 2.5 ng/μl per 10^6 cells in 20μl suspension.

To determine if the effect of Nkx2-5 is cell-autonomous, E14 cells constitutively expressing GFP (E14-GFP)[4] were mixed with iNkx2-5 cells and were induced to form EBs. Cells were induced with doxycycline from day 4 to day 6 and analyzed by FACS on day 6. For the Gata1 rescue studies, iNkx2-5 cells were infected with a lentiviral vector containing control IRES-EGFP or Gata1-IRES-EGFP genes. iNkx2-5 cells were infected, expanded, treated with 0.5 μg/ml of doxycycline and sorted for GFP positive cells to select the infected cells (yellow cells in Figure

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

6A). After two cycles of enrichment, the cells were differentiated to form EBs. In typical experiments, 20-30% of EB cells were GFP positive. Each experiment was repeated at least three times and a representative FACS profile and a graph of averaged data are shown. Bars represent standard error.

Generation of transgenic mice

Generation of Nkx2-5-EYFP transgenic[5] and Nkx2-5 heterozygous mutant[6] mice were described previously. All mice were maintained in pathogen free animal facility according to the animal care guidelines at UT Southwestern Medical Center and the University of Minnesota.

Immunohistochemistry

Induced and control Day 6 EBs were fixed in 4% paraformaldehyde for four hours at 4°C and processed for paraffin embedding. Eight micron sections were deparaffinized and serial sections were immunostained with anti-PECAM serum (1:200 dilution; Pharmingen) or stained with hematoxylin and eosin (H/E).

Methylcellulose assays

Doxycycline-inducible Nkx2-5 ES cells were cultured in standard conditions to form EBs for six days. To examine the hematopoietic differentiation of ES cells, EBs at Day 6 were then dissociated with trypsin (Cellgrow) and counted. $5x10^4$ cells were plated in 3 cm dishes in 1.5 ml methylcellulose with complete hematopoietic cytokines (Stemcell Technologies, 3434) in the presence or absence of doxycycline following the manufacturer's instructions. To examine cell fate commitment of ES cells, Day 4 EBs were treated with doxycycline (0.1 and 0.5 μg/ml) for 48

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

hours to induce Nkx2-5 expression. On Day 6, EBs with or without induction were washed, dissociated and then plated in methylcellulose without doxycycline. Colonies were scored six days after plating.

Plasmids

The reporter plasmid [pGata1(3.9)-Luc] was generated by cloning the 3.9 kb upstream *Gata1* promoter fragment into the pGL3T plasmid [7]. The *Gata1* minigene was previously described [8]. Additional constructs utilized standard molecular biological techniques. Expression of the proteins was confirmed by transfecting K562 cells and carrying out Western blot of the cell extract using anti-myc antibody (Santa Cruz). pSam2-*Gata1* or pSAM2 plasmid were generated by replacing the ubiquitin promoter of lenti FUGW[9] with sgTRE[10], and by replacing GFP with Gata1-IRES-GFP or IRES-GFP, respectively. The Gata1 cDNA sequence is from NCBI BC052653.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from samples using Trizol (Invitrogen). cDNA was synthesized using Superscript III (Invitrogen) and analyzed by real-time PCR (ABI). The Taqman probes used for the assay are listed in Supplementary Table 1 and 2. qRT-PCR experiments were carried out with at least three biological replicates and each sample was quantified in triplicate PCR reactions. Expression of genes were analyzed by singleplex comparative C_T method and normalized to that of E8.5 embryos. Real-time PCR was carried out in triplicate and data were analyzed by RQ analysis software. Error bars represent RQmin and RQmax values with 99% confidence interval calculated with RQ analysis algorithm and (ABI).

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

Transcriptional assays

All transcriptional assays were performed in the K562 cell line obtained from ATCC. $2X10^5$ cells were transfected with 250 ng of reporter gene and indicated amounts of expression vectors using Lipofectamine LTX (Roche, lipofectamine:DNA ratio=2μl :1 μg). Eight ng of pRL-TK plasmid (Promega) was co-transfected as an internal control. An empty vector (pCDNA3) was added to balance the amount of DNA transfected. Cells were harvested after 24 hours of transfection and luciferase activity was measured by Dual Luciferase Assay System (Promega). Relative luciferase activity (shown in Supplemental Figure 2) was calculated as (Luciferase activity–background)/(Renilla luciferase activity–background). Relative activity shown in Figure 5 is normalized to the transcriptional activity without the Nkx2-5 expression vector in each experimental set. Each assay was performed in triplicate and repeated at least three times. Representative data for each experiment is shown. Data represent average of triplicates and error bars indicate standard deviation. Results were analyzed by Kruskal-Wallis test (n=3).

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

References for Online Data Supplements

1. Shi X, Garry DJ. Myogenic regulatory factors transactivate the Tceal7 gene and modulate muscle differentiation. *Biochem J.* 2010.
2. Iacovino M, Hernandez C, Xu Z, Bajwa G, Prather M, Kyba M. A conserved role for Hox paralog group 4 in regulation of hematopoietic progenitors. *Stem Cells Dev.* 2009;18(5):783-792.
3. Dang SM, Kyba M, Perlingeiro R, Daley GQ, Zandstra PW. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnol Bioeng.* 2002;78(4):442-453.
4. Ismailoglu I, Yeamans G, Daley GQ, Perlingeiro RC, Kyba M. Mesodermal patterning activity of SCL. *Exp Hematol.* 2008;36(12):1593-1603.
5. Masino AM, Gallardo TD, Wilcox CA, Olson EN, Williams RS, Garry DJ. Transcriptional regulation of cardiac progenitor cell populations. *Circ Res.* 2004;95(4):389-397.
6. Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, Harvey RP. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev.* 1995;9(13):1654-1666.
7. Ferdous A, Caprioli A, Iacovino M, Martin CM, Morris J, Richardson JA, Latif S, Hammer RE, Harvey RP, Olson EN, Kyba M, Garry DJ. Nkx2-5 transactivates the Ets-related protein 71 gene and specifies an endothelial/endocardial fate in the developing embryo. *Proc Natl Acad Sci U S A.* 2009;106(3):814-819.
8. Ohneda K, Shimizu R, Nishimura S, Muraosa Y, Takahashi S, Engel JD, Yamamoto M. A minigene containing four discrete cis elements recapitulates GATA-1 gene expression in vivo. *Genes Cells.* 2002;7(12):1243-1254.
9. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science.* 2002;295(5556):868-872.
10. Agha-Mohammadi S, O'Malley M, Etemad A, Wang Z, Xiao X, Lotze MT. Second-generation tetracycline-regulatable promoter: repositioned tet operator elements optimize transactivator synergy while shorter minimal promoter offers tight basal leakiness. *J Gene Med.* 2004;6(7):817-828.

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

Supplemental Figures and Tables

Supplemental Figure 1. Luciferase activity represented in RLU.

(A-F) Pre-normalization data of those shown in Figure 5, B-G are presented in Relative Luciferase Units (luciferase light units divided by control renilla luciferase light units; RLU) to compare the absolute values of the reporter gene activity. Error bars indicate SD. See Figure 5 for statistical analysis.

Supplemental Figure 2. Mutation analysis of the Gata1 promoter.

(A) Diagram of constructs and base substitutions used in transcriptional assays and EMSA. Bolded letters indicate sequences that have homology to the Nkx2-5 binding sequence and red letters indicate the mutated residues. Mutation the G1HE region (mut1) was introduced to alter only the Nkx2-5 consensus sequence, but not the flanking Gata binding sequences. **(B-E)** Luciferase assay using mut1 (B, C) and mut 2 (D, E) constructs. Data are shown normalized to the basal activity (B, D) and as Relative Luciferase Unit (C, E). Note that the mut2 construct is repressed by Nkx2-5 in a similar dose response curve as the wild-type reporters (compare with Figures 5B, C), but mut1 is not. Panels B-E were analyzed by Kruskal-Wallis test (n=3). *:$p<0.05$ compared to sample with no Nkx2-5 expression vector (0ng). Bars without an asterisk were not significant.

Supplemental Figure 3. EMSA using NKE within the distal G1HE region shows a specific DNA binding activity.

iNkx2-5 ES cells were left untreated (lane 1) or treated with doxycycline (lanes 2-4) for 6 hr and nuclear extract was prepared. Twenty fold excess of unlabeled wild type (lane 3) or mutated (lane 4) oligonucleotides were added to test specificity. Note the induction of specific DNA

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

binding activity after 6 hrs (arrowhead). After this short (6 hr) induction, it is unlikely that secondarily induced proteins by Nkx2-5 are present in the extract. NS: non specific binding.

Supplemental Table 1

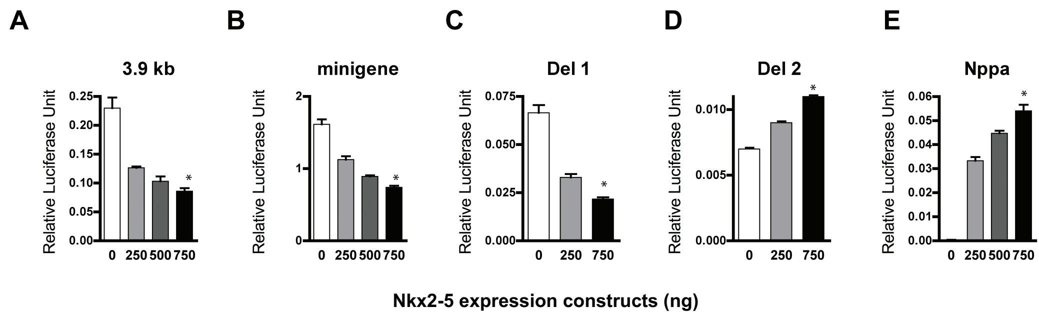
List of Taqan probe mixes used for qRT-PCR assays.

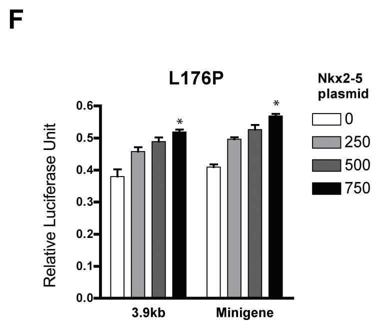
Supplemental Table 2

Sequences of custom designed Taqman probes and primers used for qRT-PCR analysis.

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

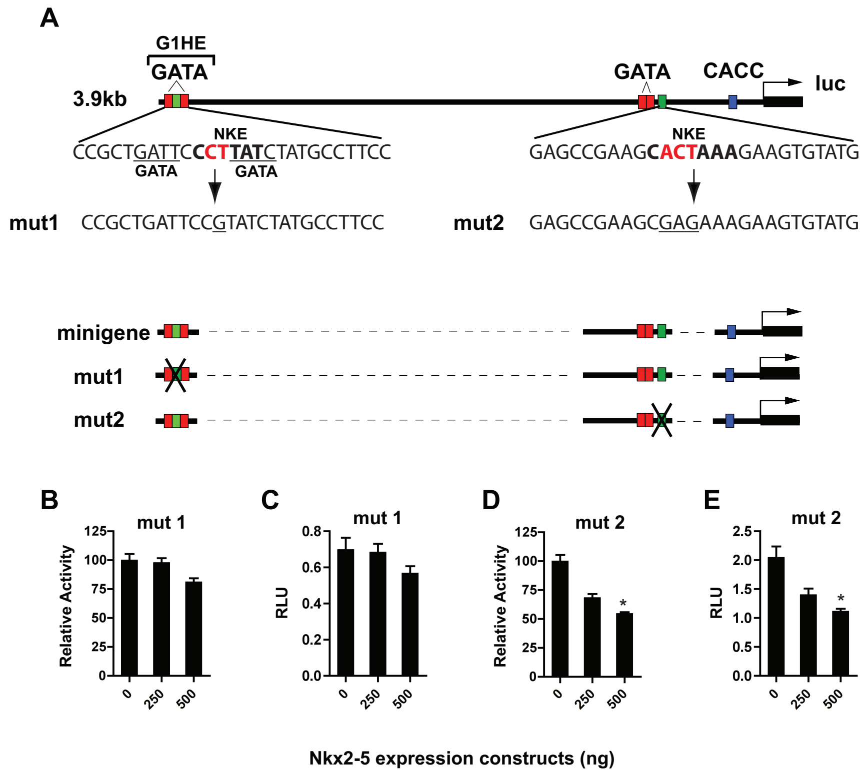
Supplemental Figure 1





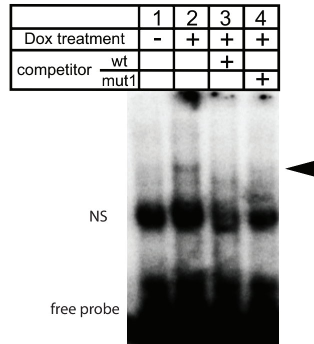
Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

Supplemental Figure 2



Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

Supplemental Figure 3



Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011

Supplemental Table 1

	Catalog number
Beta major globin	custom
Embryonic globin	custom
Etv2	Mm00468389_m1
GAPDH	4352339E
Gata1	Mm01352636_m1
Gata2	Mm00492300_m1
kdr/Flk-1	Mm00440088_m1
Klf1/Eklf	Mm00516096_m1
LMO2	Mm00493153_m1
NFE2	Mm00801891_m1
nkx2.5	Mm00657783_m1
Nppa/ANF	Mm01255747_g1
PECAM	Mm01246167_m1
Runx1	Mm50486762_m1
Scl	mm01187033_m1
Tie2	Mm01256892_m1

Supplemental Table 2

	sense	**antisense**	**probe**
Beta_Major_Glo	AGGGCACCTTTGCCAGC	GGCAGCCTGTGCAGCG	CGTGATTGTGCTGGGCCACCACCT
Emb_Globin	CCTCAAGGAGACCTTTGCTCAT	CAGGCAGCCTGCACCTCT	CAACATGTTGGTGATTGTCCTTTCT

Downloaded from http://circ.ahajournals.org/ at University of Minnesota on November 3, 2011