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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

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

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

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).

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).

References for Online Data Supplements

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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

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.

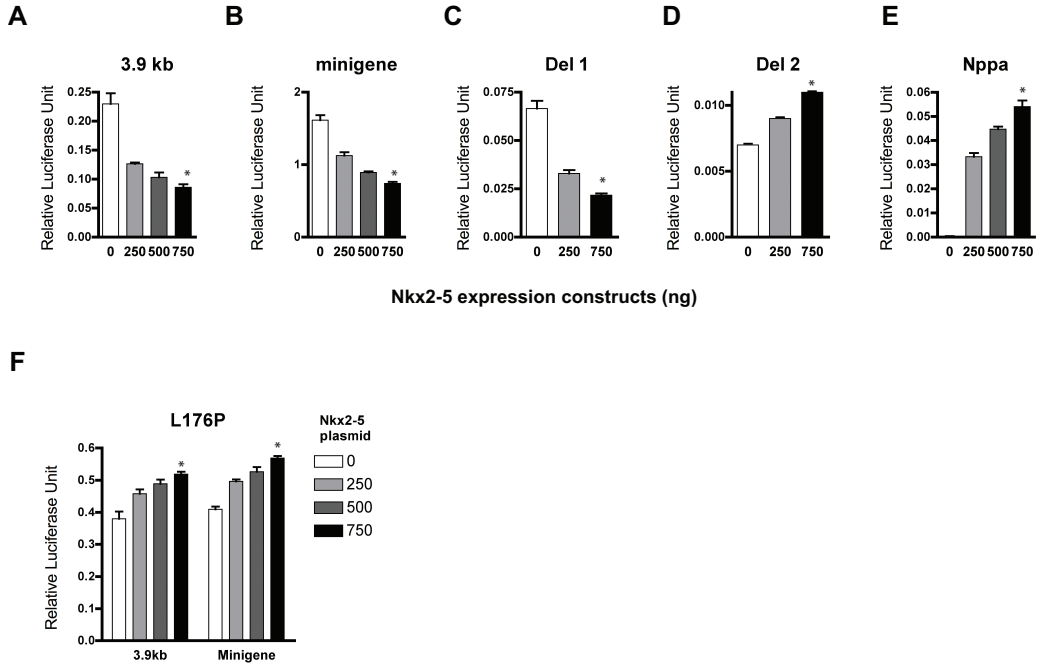
Supplemental Table 1

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

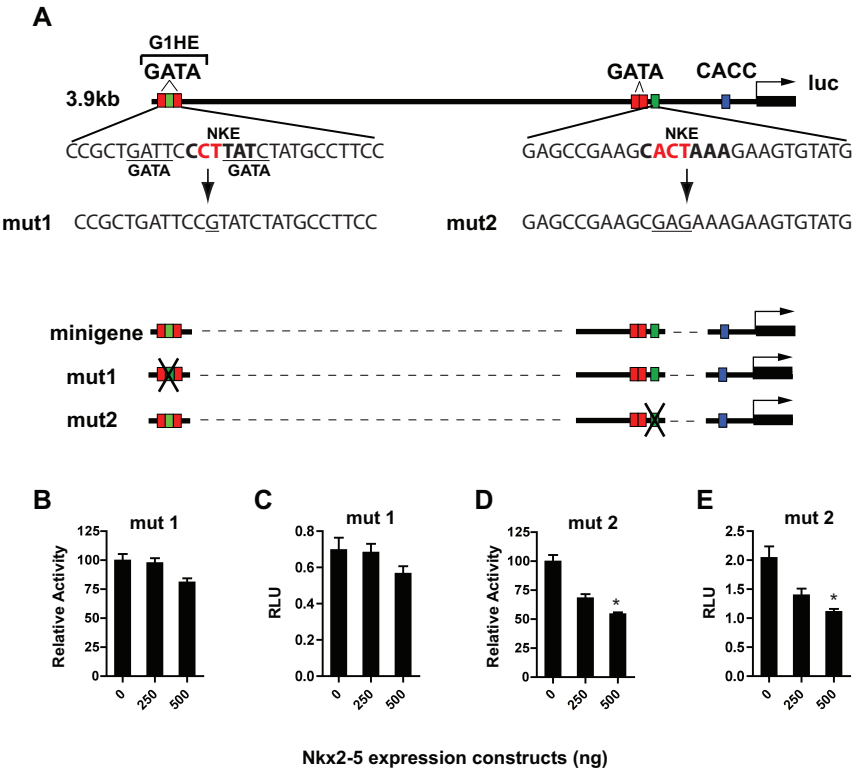
Supplemental Table 2

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

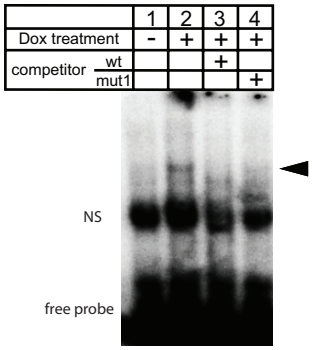
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



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