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# Induction of Id2 Expression by Cardiac Transcription Factors GATA4 and Nkx2.5

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**Abstract** Inhibitor of differentiation/DNA binding (Id) proteins function as a regulator of helix-loop-helix proteins participating in cell lineage commitment and differentiation. Here, we observed a marked induction of Id2 during cardiomyocyte differentiation from P19CL6 murine embryonic teratocarcinoma stem cells, prompting us to investigate the upstream regulatory mechanism of Id2 induction. Computer analysis of Id2 promoter and subsequent electrophoretic mobility shift assay revealed several binding sites for GATA4 and Nkx2.5 within the Id2 promoter. By further deletion and mutation analysis of the respective binding site, we identified that two motifs located at −497/−502 and −264/−270 were functionally important for Id2 promoter activation by GATA4 and Nkx2.5, respectively. Overexpression of GATA4 and/or Nkx2.5 induced not only Id2 promoter activity but also Id2 protein expression. Additionally, Id proteins significantly inhibit the GATA4 and Nkx2.5-dependent transcription, suggesting Id proteins may play a regulatory role in cardiogenesis. Collectively, our results demonstrate that GATA4 and Nkx2.5 could be one of the upstream regulators of Id2. J. Cell. Biochem. 103: 182–194, 2008. 

**Key words:** Idb2 protein; cell differentiation; transcription factors; myocytes; gene expression regulation

Inhibitor of differentiation/DNA binding (Id) proteins belong to a subfamily group of helix-loop-helix proteins (HLH) that are important regulators of cellular differentiation and proliferation. Lacking the basic amino acid residues responsible for DNA binding, Id proteins effectively prevent critical HLH transcription factors from binding to tissue-specific gene promoters, thereby acting as dominant negative regulators of HLH transcription factors [Benezra et al., 1990; Norton et al., 1998; Ruzinova and Benezra, 2003]. Four Id proteins (Id1 through Id4) are identified in vertebrates and are differentially regulated depending on cell type or stimuli [Ruzinova and Benezra, 2003]. During cardiac development, each Id transcript is widely expressed with temporal and spatial regulation. Id1, Id2, and Id3, but not Id4, are expressed in endocardial cushion, outflow tract, and valves in the developing heart in overlapping patterns [Evans and Obrien, 1993; Jen et al., 1996]. Id transcript is also highly expressed in intact heart tissue as well as in freshly isolated cardiac muscle cells from neonatal and adult rat heart [Springhorn et al., 1992]. Moreover, double or triple-Ids knockout mouse embryos have multiple cardiac abnormalities including ventricular septal defects, endocardiac cushion defects, and myocardial wall defects, and die during mid-gestation [Fraidenraich et al., 2004]. These studies suggest a pivotal role of Id protein in cardiac development.

GATA4 and Nkx2.5 are transcription factors essential for heart development and act as early pre-cardiac cell markers. GATA4 is a member of the zinc finger GATA family (GATA1 through GATA6) and is expressed predominantly in the heart, digestive organs, and the extra-embryonic endoderm [Heikinheimo et al., 1994; Laverriere et al., 1994]. GATA4 binds to the WGATAR

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motif within the promoter region of its target genes and regulates structural and regulatory gene expression such as ANF, BNP, cardiac troponin C, and α-MHC in cardiac muscle cells [Ip et al., 1994; Molkentin et al., 1994; McBride and Nemer, 2001]. Nkx2.5/Csx, a NK class homeodomain protein, is also expressed predominantly in the heart, cardiac precursor cells, and regulates cardiac specific gene expression [Durocher et al., 1996]. In mice, targeted disruption of GATA4 or Nkx2.5 results in severe cardiac developmental defects causing embryonic lethality, suggesting critical role of GATA4 and Nkx2.5 in cardiac development [Lyons et al., 1995; Molkentin et al., 1997].

P19CL6 cells, a well-established subclone derived from P19 mouse embryonal teratocarcinoma stem cell line [HabaraOhkubo, 1996], differentiate into beating cardiomyocytes by 1% DMSO. Owing to the differentiation efficiency (>80%) as compared to the P19 cells [Mcburney et al., 1982] and the characteristics mimicking the in vivo cardiac gene expression patterns, P19CL6 cells are widely used to study the molecular mechanisms of cardiomyocyte differentiation in vitro. In this study, we observed that Id2 is markedly upregulated during cardiomyocyte differentiation from P19CL6 cells, which prompted us to investigate the involvement of cardiac transcription factors in Id2 regulation. The induction of Id2 could, in concert with the inhibitory role of Ids in GATA4 and Nkx2.5-dependent transcription, be implicated in fine regulation of cardiogenesis.

## METHODS

### Cell Culture and Differentiation

P19CL6 cells were kindly provided by Dr. I. Komuro (Chiba University Graduate School of Medicine, Japan) and cultured as described previously [HabaraOhkubo, 1996]. In brief, P19CL6 cells were cultured in α-minimal essential medium containing heat-inactivated 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% $CO_2$ atmosphere at 37°C. To induce differentiation, $3.7 \times 10^5$ cells were plated in a 60-mm culture dish with 1% DMSO-containing growth medium and the medium was changed every 2 days. Spontaneous beating was observed 11–12 days after DMSO treatment. H9c2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% $CO_2$ atmosphere at 37°C.

### Materials

Cell culture reagents were purchased from GIBCO BRL (Rockville, MD). DMSO and protease inhibitor cocktail were from Sigma–Aldrich. BMP4 was obtained from R&D Systems. Anti-GATA4, anti-Nkx2.5, anti-Id1, anti-Id2, anti-Id3, and anti-Actin antibodies were purchased from Santa Cruz Biotechnology (CA). Anti-HA antibody was from Upstate (Charlottesville, VA). Anti-sarcomeric MyHC (MF20) antibody was obtained from Iowa University.

### Plasmids and Transfection

Mouse Id2 promoter luciferase reporter plasmids (−2274/+59Id2-Luc, −574/+59Id2-Luc, −327/+59Id2-Luc, −126/+59Id2-Luc) were kindly provided by Dr. Yoshifumi Yokota (University of Fukui, Japan) [Karaya et al., 2005]. −1403/+59Id2-Luc, −2274/mtG4-Luc, and −2274/mtN4-Luc plasmid was prepared by PCR-based cloning method with appropriate primers, using −2274/+59Id2-Luc plasmid as a template. PCR product was ligated into the pCR2.1 TA vector (Invitrogen) and subcloned into the pGL2 vector using *Kpn*I and *Xho*I restriction sites. HA-tagged GATA4 and HRT1 were generated by subcloning the corresponding PCR product into the pcDNA3.1TOPO expression vector. The GATA4 expression vector and ANF-Luc reporter plasmid were kindly provided by Dr. Mona Nemer (Institut de Recherches Cliniques de Montreal, Canada). The Nkx2.5 expression vector was kindly provided by Dr. Tae-gyun Kim (Korea Food and Drug Administration, Korea, [Kim et al., 2004]). MEF2C-Luc reporter plasmid was kindly provided by Dr. Eric Olson (Univ. of Texas Southwestern Medical Center, USA). All constructs were verified by DNA sequencing. For the transactivation assay, the luciferase reporter plasmid was co-transfected with β-galactosidase using lipofectamine (Invitrogen), following manufacturer's protocol. Twenty hours post transfection, the cells were lysed with reporter lysis buffer (200 μl/well, Promega) and luciferase activity was measured using the luciferase reporter assay system (Promega). β-galactosidase activity was used to normalize transfection efficiency.

### RT-PCR and Semi-Quantitative PCR

Total RNA was extracted from cultured cells using Trizol (Invitrogen) according to the manufacturer's instructions. The first-strand cDNA synthesis was performed with 1 μg of extracted RNA, random primer (Amersham Pharmacia), and reverse transcriptase MMLV (Invitrogen). For semi-quantitative PCR, the following oligonucleotide primers were used: Id1, 5′-GGTCGCCAGTGGCAGTGCCGC-3′ (forward), 5′-ACATGCTGCAGGATCTCCA-3′ (reverse): Id2, 5′-GCATCCCCCAGAACAAGA-AGGT-3′ (forward), 5′-CCAGGCCGGAGAACAAGACAC-3′ (reverse): Id3, 5′-GGTGCGCGGCTGCTAC-GAG-3′ (forward), 5′-CAGGCCACCCAAGTT-CAGTCC-3′ (reverse). Primers for GATA4, Tbx5, and α-MHC were described previously [Hidai et al., 2003]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control: 5′-AGACAAGATGGTGAA-GGTCGG-3′ (forward), 5′-TCATGAGCCCTTC-CACGATGC-3′ (reverse). PCR products were analyzed on 1% agarose gels.

### Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared 24 h after transfection as described previously [Wadman et al., 1997]. Briefly, cells were scraped in 400 μl of buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5% NP-40, protease inhibitor cocktail, and 0.5 mM PMSF) and incubated on ice for 15 min. After centrifugation, the cell pellet was resuspended in 150 μl of buffer C (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% v/v Glycerol, protease inhibitor cocktail, and 0.5 mM PMSF) and incubated on ice with vigorous agitation for 40 min. Nuclear extracts were recovered by centrifugation for 15 min at 13,000 rpm at 4°C and protein concentration was determined by the Bradford method (Bio-Rad).

For EMSA, the following probes derived from dHAND (dHAND-GBE) and ANF (ANF-NKE) promoter were used for GATA4 and Nkx2.5 binding assay [Kasahara et al., 2001; Kathiriya et al., 2004]: dHAND-GBE, 5′-TCGAGGTAAT-TAACTGATAATGGTGC-3′: ANF-NKE, 5′-TCA-CACCTTTGAAGTGGGGGCCT-3′. Approximately 100 pM of sense or antisense oligonucleotides were end-labeled with [$\gamma$-$^{32}$P] ATP by T4 polynucleotide kinase for 1 h at 37°C. Labeled and purified sense-antisense oligos were combined and annealed in 5× annealing buffer (Amersham Pharmacia Biotech). Nuclear extracts (5 μg) and labeled oligoprobes were used in the gel shift assay. A 50-fold molar excess of unlabeled competitor was used for the competition experiments. For the supershift assay, 2–3 μg of antibodies were added to the reaction mixtures prior to radiolabeled probe addition. Binding mixtures were run on a 4% non-denaturing acrylamide gel and visualized by autoradiography.

### Western Blotting

Western blotting was performed as described previously [Lim et al., 2005]. Whole cell extract (30 μg) was subjected to SDS–PAGE and transferred to a nitrocellulose membrane. After blocking with 5% non-fat dry milk, the blot was sequentially incubated with primary antibody and horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive bands were detected using an enhanced chemilluminescence detection kit.

### Immunofluorescence Assay

Cells were fixed, quenched, and then permeabilized as described previously [Lim et al., 2005]. After blocking with 2% (w/v) BSA for 1 h, the sample was incubated with a rabbit anti-Id2 polyclonal antibody (1:100 dilution) at 4°C overnight. Cells were washed and incubated with anti-rabbit rhodamine-conjugated secondary antibody for 1 h at room temperature. Nuclei were stained with DAPI.

### Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed as described previously with minor modifications [Gonda et al., 2003]. For cross-linking between protein and DNA, P19CL6 cells on the differentiation day 4 were treated with 1% formaldehyde in the medium for 10 min at 37°C. After washing with ice-cold PBS containing protease inhibitors, cells were resuspended in ChIP sonication buffer (1% Triton X-100, 50 mM Tris pH 8.1, 150 mM NaCl, 0.1% Na-Deoxycholate, 5 mM EDTA and protease inhibitors cocktail), incubated on ice for 20 min, and then sonicated on ice to shear DNA to 500–1,000 bp fragments. The sonicated lysates were immunoprecipitated with 5 μg of anti-GATA4 antibody or normal IgG antibody. For the negative control, no antibody was added. Antibody–protein–DNA complexes were isolated with protein A/G agarose and

were sequentially washed with low salt buffer, high salt buffer, LiCl buffer, and TE buffer [Gonda et al., 2003]. Immunocomplexes were eluted and heated at 65°C for 6 h to reverse the formaldehyde cross-links. After treatment with proteinase K at 55°C for 1 h, the samples were extracted with phenol-chloroform, and precipitated with ethanol. Precipitated DNA was resuspended in TE and amplified by PCR using the following primer sets: 5′-TGTGCAAACCCCACTAATGA-3′ (forward), 5′-CGCTTTTGGGAAGTCACATT-3′ (reverse).

### Statistical Analysis

All values were expressed as means ± SD. Statistical comparison between experimental groups was performed using student *t*-test. *P*-values $< 0.05$ were considered to be statistically significant.

## RESULTS

### Induction of Id2 Expression in Differentiating P19CL6 Cells

We examined the expression patterns of the Id genes at 4-day intervals during P19CL6 differentiation using semi-quantitative RT-PCR and Western blotting. We monitored cardiac-specific gene expression for the progression of differentiation (Fig. 1A). In the differentiating medium containing 1% DMSO, P19CL6 cells expressed GATA4, Nkx2.5, and Tbx5 transcript within 4–8 days and the cardiac contractile gene α-myosin heavy chain (α-MHC) on day 12 (Fig. 1A). Bone morphogenetic protein (BMP)-4 required for cardiomyocyte differentiation was induced at the early phase of differentiation but decreased as the differentiation progressed [Monzen et al., 2001; Nakamura et al., 2003]. Id1 and Id2 were also prominently induced at the early stage of differentiation, but Id1 gradually decreased in contrast to Id2 maintaining relatively high level until the later differentiation stage (Fig. 1A). Id3 was slightly upregulated but kept low expression. The mRNA expression was reflected on protein expression (Fig. 1B). Consistently, the population of Id2-positive cells was far increased at differentiation day 4 compared with control (Fig. 1C).

### Id2 Promoter Activation by Cardiac Transcription Factors GATA4 and Nkx2.5

Because especially Id2 showed prominent induction in our cardiomyocyte-specific

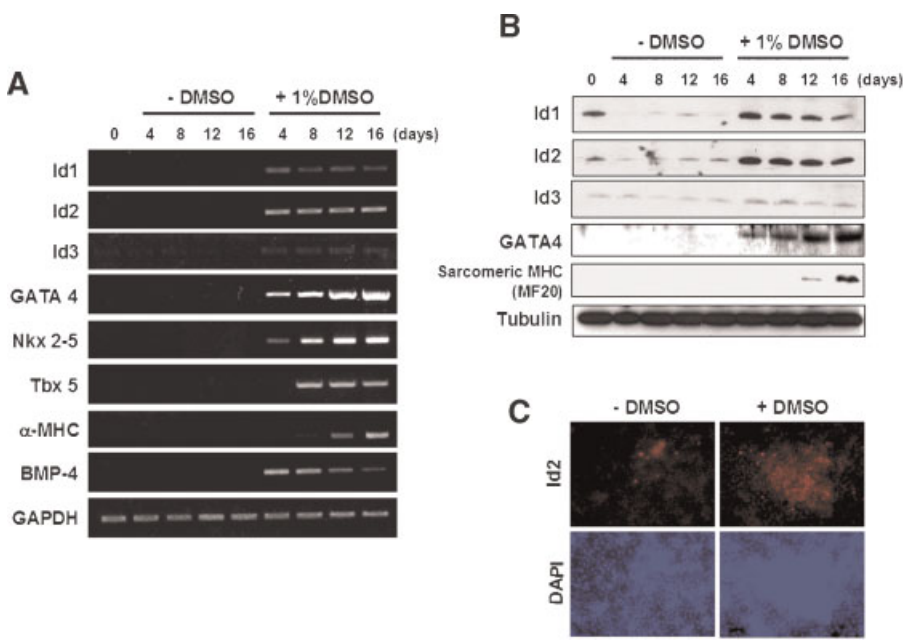


**Fig. 1.** Induction of Id expression during cardiomyocyte differentiation from P19CL6 cells. Cells were either induced to differentiate into cardiomyocytes by incubation with 1% DMSO or maintained in a proliferating state without DMSO treatment. Cell lysates were prepared at 4-day intervals as indicated. Total RNA and protein were extracted from cell lysates and aliquots (1 μg of total RNA and 30 μg of protein extract) were used for semi-quantitative RT-PCR (**A**) or Western blotting (**B**). GAPDH and tubulin were used as internal loading controls for PCR and western blotting, respectively. **C**: Immunofluorescence staining indicates Id2 protein expression. Cells were cultured for 4 days with (right) or without (left) 1% DMSO and stained with Id2 antibody (red). Nuclei were stained with DAPI (blue).

differentiation system, we first investigated the relevance of cardiac transcription factors to the Id2 expression. While analyzing the 5′ flanking region of the Id2 promoter (Accession no. AC116680), we found several potential binding sites for two important cardiac transcription factors GATA4 and Nkx2.5 within the Id2 promoter regions, ranging from approximately −2 kb relative to the transcriptional initiation site (Table I and Fig. 2A). The cells under cardiomyocyte differentiation showed significant elevation of the reporter construct containing the −2274 to +59 region of the Id2 promoter (Id2-Luc) (Fig. 2B). Moreover, GATA4 or Nkx2.5 overexpression through transfection activated the Id2-Luc activity in a dose-dependent manner in the proliferating P19CL6 cells (Fig. 2C), and co-transfection of GATA4 and Nkx2.5 synergistically increased the Id2 promoter activity up to sixfold (Fig. 2D). Because proliferating P19CL6 cells do not express any of the cardiac factors capable of affecting Id expression such as BMP (Fig. 1A), we could demonstrate the direct effect of GATA4 and Nkx2.5 on Id2 promoter activation. Furthermore, employing the biological characteristic of hairy-related transcription factor (HRT)-1 acting as a repressor for GATA protein [Kathiriya et al., 2004], we confirmed that Id2 is under the control of GATA4. HRT1 completely prevented the GATA4-induced Id2 promoter activation as well as the ANF promoter activation used as a positive control (a representative target gene regulated by GATA4 and Nkx2.5) (Fig. 2E). Taken together, these results suggest that Id2 promoter could be activated by cardiac transcription factors GATA4 and Nkx2.5.

### Id2 Protein Induction by Transient Overexpression of GATA4 and Nkx2.5

To directly demonstrate that GATA4 and Nkx2.5 could induce Id2 protein expression, we transiently overexpressed GATA4 and Nkx2.5 in proliferating P19CL6 cells (Fig. 3A). The GATA4 or Nkx2.5 alone was enough to induce Id2 protein expression and simultaneous overexpression of both led to further induction. The similar results were observed in rat cardiomyocyte precursor cell line H9c2 (Fig. 3B), suggesting the observation that GATA4 and Nkx2.5 are involved in Id2 regulation is not specific to P19CL6 cells.

### GATA4 and Nkx2.5 Bind to Their Putative Binding Element Within the Id2 Promoter

As shown in Figure 2A and Table I, we found several putative binding sites for GATA4 and Nkx2.5 within the Id2 promoter. To verify the binding affinity of these sites, we performed EMSAs with the nuclear extract (NE) prepared from HA-GATA4- or Nkx2.5-overexpressing cells. Using the previously identified probes from dHAND promoter (dHAND-GBE) and ANF promoter (ANF-NKE) as positive probes, we first examined whether the unlabeled Id2-GBEs and Id2-NKEs probes (see Table I) could compete with the positive probes. As expected,

**TABLE I. Putative GATA4 and Nkx2.5 Binding Sites Within Id2 Promoter**

| Probe | Sequence (5′ → 3′) | Location |
|---|---|---|
| Id2-GBE1 | cagcgctcaTTATCAtccagccca | −1812/−1807 |
| Id2-GBE2 | gggggactcAGATAGtaaatcact | −1503/−1498 |
| Id2-GBE3 | taaaaacagTGATAAgctgtcagt | −941/−936 |
| Id2-GBE4 | agctttcctTTATCTtaaaagaag | −502/−497 |
| Id2-GBE5 | agggatgccCGATTGagcggccag | −78/−73 |
| mt Id2-GBE1 | cagcgctcaTTTGCAtccagccca | −1812/−1807 |
| mt Id2-GBE2 | gggggactcAGCAAGtaaatcact | −1503/−1498 |
| mt Id2-GBE3 | taaaaacagTGCAAAgctgtcagt | −941/−936 |
| mt Id2-GBE4 | agctttcctTTTGCTtaaaagaag | −502/−497 |
| Id2-NKE1 | tgccacctaCACTGAAgggcacaga | −814/−808 |
| Id2-NKE2 | aaatgtagtTCCAGTGtgcaaaaccc | −772/−766 |
| Id2-NKE3 | aagaaggaaCACTACAaatgtgact | −484/−478 |
| Id2-NKE4 | caattgcaCACTCAGgccccctgg | −270/−264 |
| Id2-NKE5 | ccagcgcaCACTGTActcaatttg | −242/−236 |
| mt Id2-NKE1 | tgccacctaCGATGAAgggcacaga | −814/−808 |
| mt Id2-NKE3 | aagaaggaaCGATACAaatgtgact | −484/−478 |
| mt Id2-NKE4 | caattgcaCGATCAGgccccctgg | −270/−264 |

*Consensus GBE: 5′-(A/T)GATA(A/G)-3′, consensus NKE: 5′-TNNAGTG-3′.
Five putative binding sites of GATA4 and Nkx2.5 [Simon, 1995] were selected based on sequence homology [Harvey, 1996] (as shown in underlined capitals). Sense or antisense sequences of oligonucleotide probe used for EMSA were provided according to their location. The following were the sequences of the mutant probes for the competition assay: mt Id2-GBEs, GATA → GCAA; mt Id2-NKEs, TNNAGTG → TNNATCG. Asterisk indicates consensus GBE and consensus NKE.

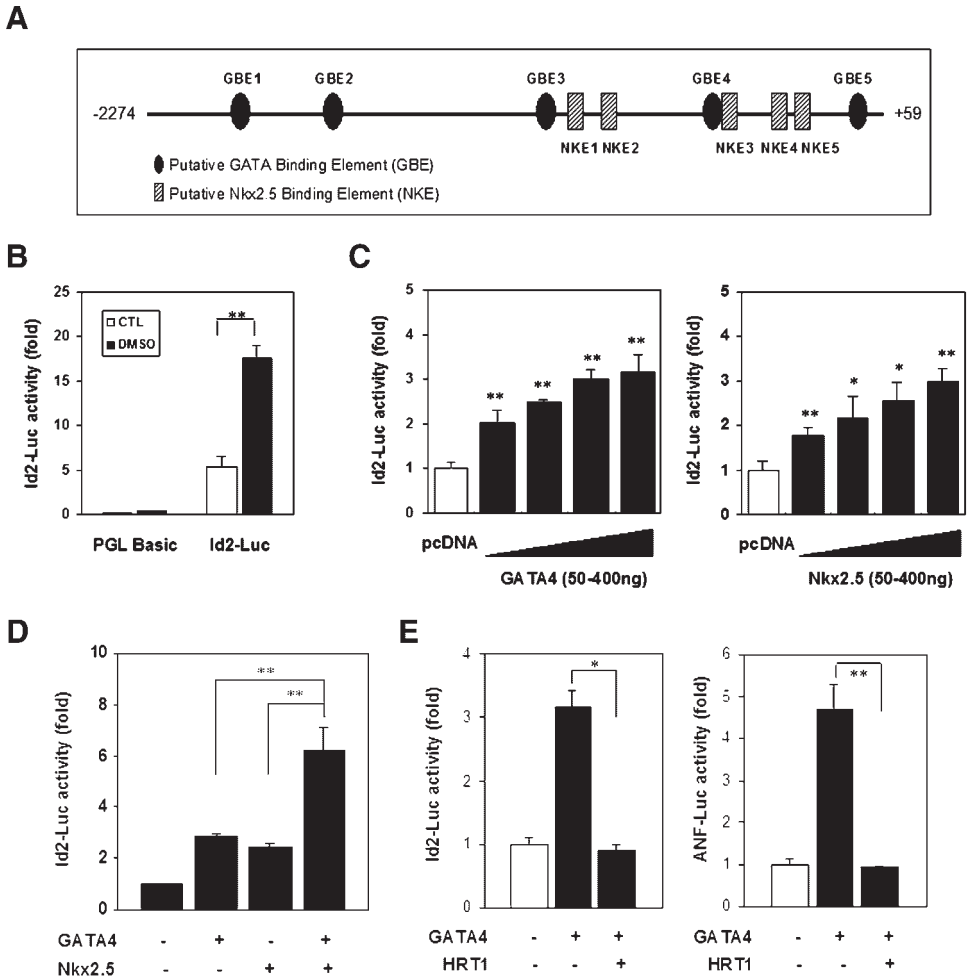


**Fig. 2.** Id2 promoter activation by GATA4 and/or Nkx2.5. **A**: Schematic description of relative locations of putative GATA4 (●, GBE1 through GBE5) and Nkx2.5 (□, NKE1 through NKE5) binding sites within the Id2 promoter (−2274/+59). **B**: P19CL6 cells were maintained with or without 1% DMSO for 3 days, and then cotransfected with PGL basic or Id2-Luc (−2274/+59) reporter plasmid (1 μg/well), together with β-galactosidase gene (50 ng/well). After 20 h of incubation, luciferase activity was examined and normalized to β-galactosidase activity $^{**}P < 0.01$. **C**: Cells were co-transfected with Id2-Luc (1 μg/well), β-galactosidase (50 ng/well), and with either GATA4 (**left**) or Nkx2.5 (**right**) expression vector in a dose-dependent manner (50, 100, 200, and 400 ng). After 20 h of incubation, luciferase activity was examined and normalized to β-galactosidase activity. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with empty vector (pcDNA)-transfected cells. **D**: Cells were co-transfected with Id2-Luc (1 μg/well) and β-galactosidase (50 ng/well) together with 200 ng of each GATA4 and Nkx2.5 $^{**}P < 0.01$. **E**: Cells were co-transfected with the Id2-Luc (left, 1 μg/well) or ANF-Luc (right, 1 μg/well) and β-galactosidase (50 ng/well) together either with 400 ng of GATA4 or 200 ng of HRT1. Relative luciferase activity was determined by statistical analysis of three independent experiments. Total amount of DNA transfected was kept constant using pcDNA3.1 empty vector. $^{*}P < 0.05$, $^{**}P < 0.01$.

the dHAND-GBE and ANF-NKE probe formed a binding complex with HA-GATA4 and Nkx2.5, respectively (lane 11 in Fig. 4A and Fig. 5A), which was prevented by respective cold self-probe (lane 12 in Figs. 4A and 5A) and also supershifted by respective antibody addition (lane 18 in Figs. 4A and 5A). Unlabeled Id2-GBE1 through Id2-GBE4 (lane 13–16 in Fig. 4A) and Id2-NKE1, -3, and -4 (lane 13, 15, and 16 in Fig. 5A) disrupted the protein-probe complex formation. Id2-GBE5 (lane 17 in Fig. 4A) and Id2-NKE2 and -5 (lane 14 and lane 17 in Fig. 5A) did not show the disrupting effect. Consistent with the competition experiments, Id2-GBE1 through Id2-GBE4 probes bound HA-GATA4 (Fig. 4B) and Id2-NKE1, -3, and -4 probes bound Nkx2.5 (Fig. 5B). These complexes were prevented by cold self probe (wt) as well as cold positive probe (dHD in Fig. 4B and ANF in Fig. 5B) but not by the

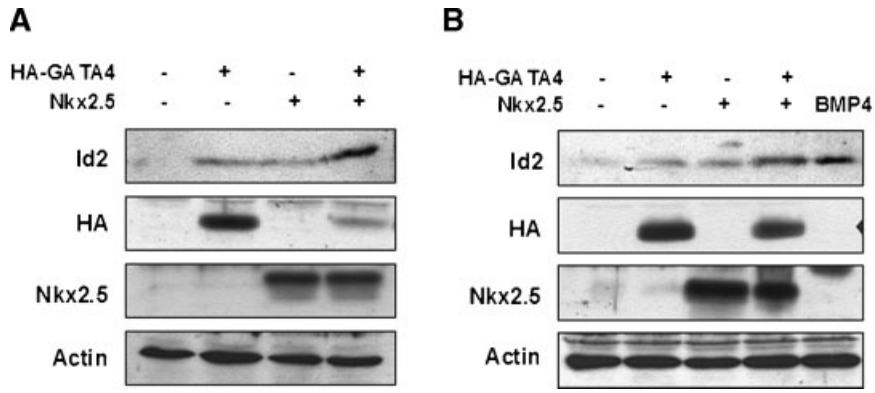


**Fig. 3.** Ectopic expression of GATA4 and Nkx2.5 induces Id2 protein expression. P19CL6 (**A**) or H9c2 (**B**) cells were transfected with either HA-GATA4 and/or Nkx2.5. Total amount of DNA transfected was kept constant using pcDNA3.1 empty vector. After 20 h of further incubation, cells were harvested and whole cell lystes were extracted. BMP4 (10 ng/ml) was used as a positive control for Id2 induction. Actin was used as a loading control.

mutated self probe (mt), and supershifted by respective antibodies.

### Identification of Functional Binding Sites for GATA4 and Nkx2.5 Within the Id2 Promoter

Next, to determine which binding sites out of the selected GBEs and NKEs are functionally important for Id2 transcription, we sequentially deleted the binding sites and compared their effect on the Id2 promoter activity in GATA4 (Fig. 6A) and Nkx2.5-transfected P19CL6 cells (Fig. 6B). Deletion of neither GBE1 through GBE3 nor NKE1 through NKE3 affected Id2 promoter activation in response to respective GATA4 and Nkx2.5 overexpression; GBE4 (−497/−502) or NKE4 (−264/−270) alone was sufficient to exert full activity of the Id2 promoter, but the respective deletion significantly abolished the Id2 promoter activity. To further demonstrate the importance of GBE4 (−497/−502) and NKE4 (−264/−270), we selectively mutated only the GBE4 or NKE4 in Id2-Luc as shown in underlined characters in Figure 6C (Id2-Luc/mG4, AGATAA → AGCAAA; AA; Id2-Luc/mN4, CTGAGTG → CTGATCG) and examined their effect on the Id2 promoter activity in response to GATA4 or Nkx2.5 overexpression. As expected, the mutations significantly impaired Id2 promoter activity (Fig. 6C). Additionally, we confirmed that GATA4 extracted from differentiating P19CL6 cells on day 11 exhibits binding affinity to Id2-GBE4 probe (Fig. 7A), suggesting GATA4 intrinsically participate in Id2 transcription during differentiation through GBE4 (−497/−502) motif. To further demonstrate this, we extracted chromosomal DNA on cardiomyocyte differentiation day 6 and performed ChIP assay with anti-GATA4 antibody (Fig. 7B). Although the band was not prominent, we could observe the PCR product comprising GBE4, indicating that GATA4 indeed adheres to endogenous Id2 promoter during cardiomyocyte differentiation. These results suggest that two motifs at −497/−502 and −264/−270 are functionally important for Id2 induction by GATA4 and Nkx2.5, respectively.

### Id Proteins Suppress GATA4 and Nkx2.5-Dependent Transcription

Id proteins are known to regulate cell lineage commitment and differentiation inhibiting tissue-specific bHLH-dependent transcription. To investigate the functional role of Id proteins in cardiomyocyte differentiation, we examined the effect of Ids on GATA4 and Nkx2.5-dependent transcription in P19CL6 cells. As shown in Figure 8, transient overexpression of Ids significantly suppressed ANF and MEF2C promoter activation induced by GATA4 and Nkx2.5, suggesting that Id proteins have an inhibitory role in cardiac transcription factor-dependent transcription.

Taken together, we demonstrate a new regulatory mechanism of Id2 induction by cardiac transcription factors GATA4 and Nkx2.5 along with the identification of their functional binding sites within the Id2 promoter, suggesting GATA4 and Nkx2.5 could be one of the upstream regulators of Id2.

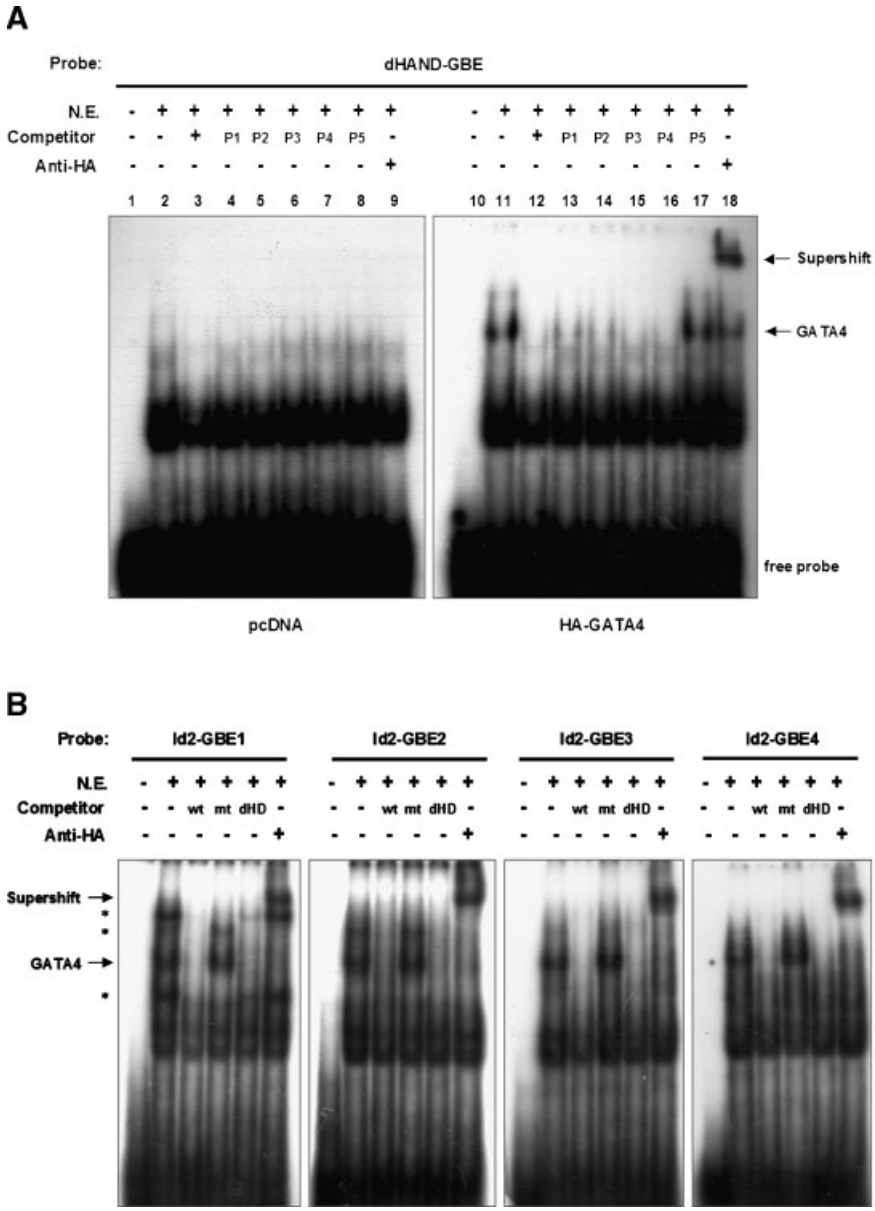


**Fig. 4.** GATA4 binds to the Id2 promoter. **A**: dHAND-GBE probe was incubated with nuclear extracts (NE) prepared from P19CL6 cells expressing pcDNA empty vector (**left**) or HA-GATA4 (**right**). A fiftyfold molar excess of cold self (**lane 3** and **lane 12**) or cold Id2-GBE probes (**lanes 4–8 and 13–17**, P1 through P5 sequentially correspond to Id2-GBE1 through Id2-GBE5 in Table I) were used for competition. The binding complex was supershifted by adding anti-HA antibody (**lane 18**). **B**: Id2-GBE1 through Id2-GBE4 probes were respectively incubated with the NE prepared from P19CL6 cells expressing HA-GATA4 to induce complex formation. A fiftyfold molar excess of cold self (wt) or cold mutant probes (mt, see Table I) were used for competition. Commonly, cold dHAND-GBE probe (dHD) was used as a positive competitor. Asterisks indicate non-specific bands.

## DISCUSSION

Here we have demonstrated that Id2 expression is induced by cardiac transcription factor GATA4 and Nkx2.5. This conclusion is based on following observations: (1) overexpression of GATA4 and/or Nkx2.5 increased Id2 promoter activity and protein expression, (2) Id2 has several GATA4 and Nkx2.5 binding sites in its promoter, and (3) two motifs located at −497/−502 and −264/−270 are functionally important for Id2 promoter activation by GATA4 and Nkx2.5, respectively. Because proliferating P19CL6 cells do not express any BMP family members, a strong inducer of Ids [Hollnagel et al., 1999; Nakashima et al., 2001; Korchynskyi

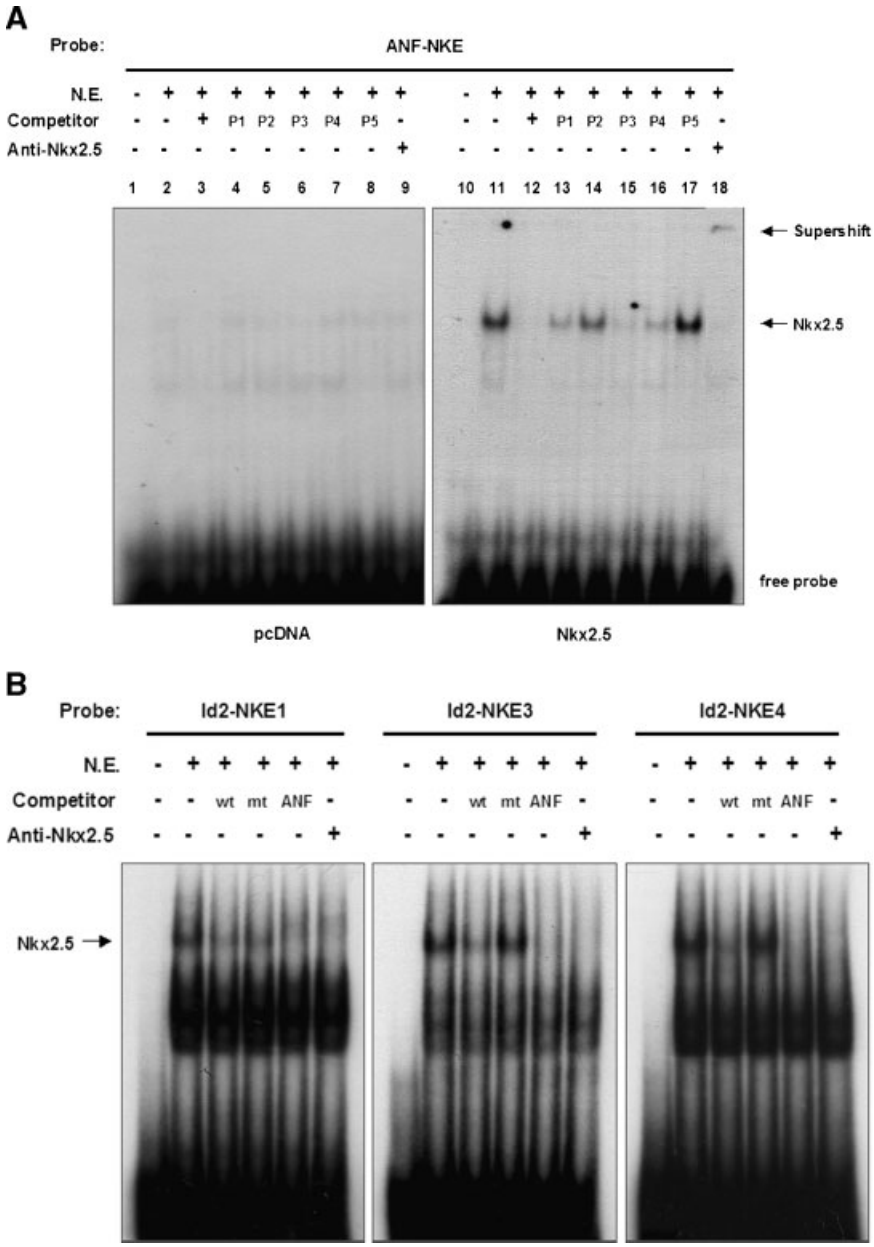


**Fig. 5.** Nkx2.5 binds to the Id2 promoter. **A**: The same approach as in Figure 4 was employed to demonstrate binding of Nkx2.5 to the Id2 promoter. Nuclear extract (NE) prepared from P19CL6 cells expressing pcDNA (left) or Nkx2.5 (right) was incubated with positive probe (ANF-NKE) for complex formation. A fiftyfold molar excess of cold self (**lanes 3**, **12**) or cold Id2-NKE probes (**lanes 4–8 and 13–17**, P1 through P5 sequentially correspond to Id2-NKE1 through Id2-NKE5 in Table I) were used for competition. The binding complex was supershifted by adding anti-Nkx2.5 antibody (**lane 18**). **B**: Id2-NKE1, -3, and -4 probes were respectively incubated with the NE prepared from P19CL6 cells expressing Nkx2.5. A fiftyfold molar excess of cold self (wt) or cold mutant probes (mt, see Table I) were used for competition. Commonly, cold ANF-NKE probe (ANF) was used as a positive competitor.

and ten Dijke, 2002; Lopez-Rovira et al., 2002], we could exclude the influence of BMPs and demonstrate a more direct and pure effect of GATA4 and Nkx2.5 on Id2 regulation. Additionally, the stimulating effect was observed in H9c2 cells, suggesting the effect is not specific to P19CL6 cell line. Interestingly, we observed a small additive effect of GATA4 and Nkx2.5 on BMP4-induced Id2 promoter activation (data not shown), raising a possibility that GATA4 and Nkx2.5 could be involved in the Id2 expression in cooperation with the BMP4-dependent signaling pathway. As a matter of fact, GATA4 and Smad1/4 have been reported to

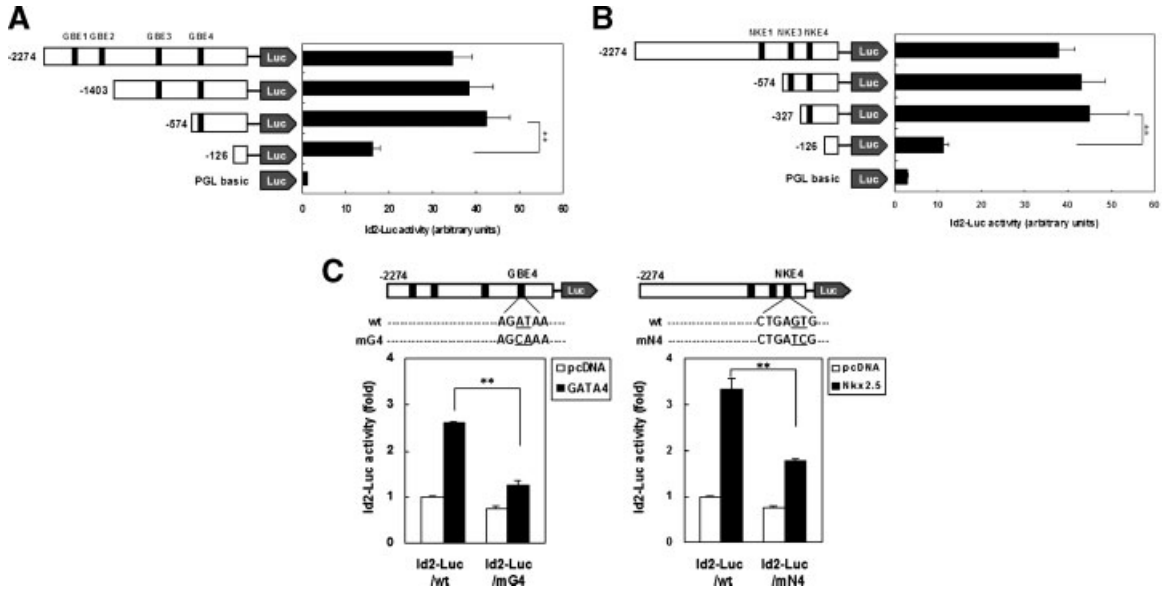


**Fig. 6.** Identification of the functional binding sites for GATA4 and Nkx2.5 within the Id2 promoter. **A**: For the determination of functional GATA4 binding site out of four candidates (GBE1 through GBE4) showing binding affinity to GATA4, the sites were sequentially deleted as depicted. Each deletion mutant (1 μg/well) was cotransfected along with HA-GATA4 (400 ng/well) and β-galactosidase (50 ng/well) into proliferating P19CL6 cells. Relative luciferase activity was determined by statistical analysis of three independent experiments $^{**}P < 0.01$. **B**: Similarly, for the determination of functional Nkx2.5 binding site, the three NKEs exhibiting binding affinity to Nkx2.5 were sequentially deleted, cotransfected with Nkx2.5 and β-galactosidase, and estimated as above $^{**}P < 0.01$. **C**: Cells were cotransfected with Id2-Luc/wt, Id2-Luc/mG4, or Id2-Luc/mN4 (1 μg/well) and β-galactosidase (50 ng/well) together with 400 ng of pcDNA, GATA4 (**left**), or Nkx2.5 (**right**). Total DNA amount transfected was kept constant using pcDNA empty vector. $^{**}P < 0.01$.

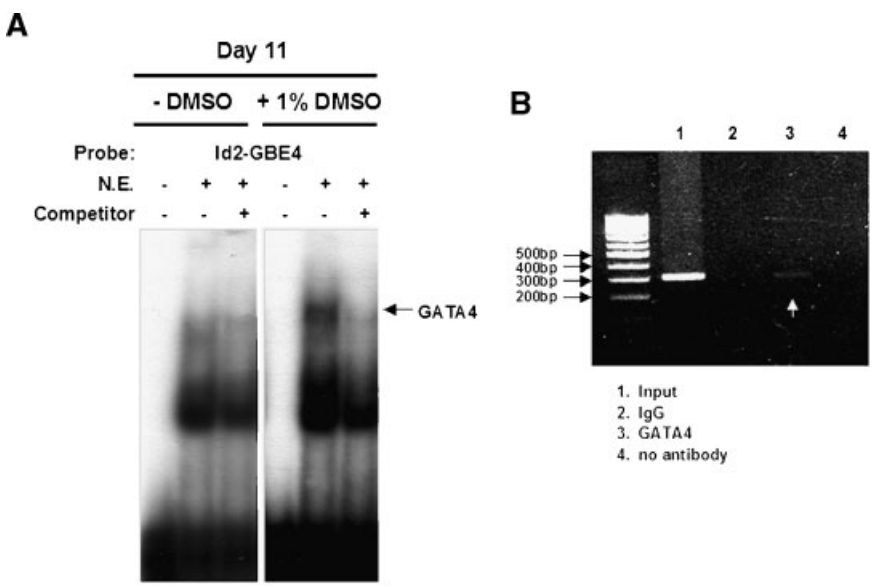


**Fig. 7.** GATA4 endogenously binds to Id2 promoter during differentiation. **A**: Nuclear extract from differentiating P19CL6 cells on day 11 was used for EMSA with Id2-GBE4 probe. **B**: ChIP assay was performed with P19CL6 cells on differentiation day 6. Immunoprecipitation was performed with 5 μg of anti-GATA4 antibody (**lane 3**) or normal IgG (**lane 2**), and no antibody was used for the negative control (**lane 4**). Precipitated DNA was amplified by PCR with a primer set containing GBE4. Diluted sheared genomic DNA was used for input (**lane 1**).

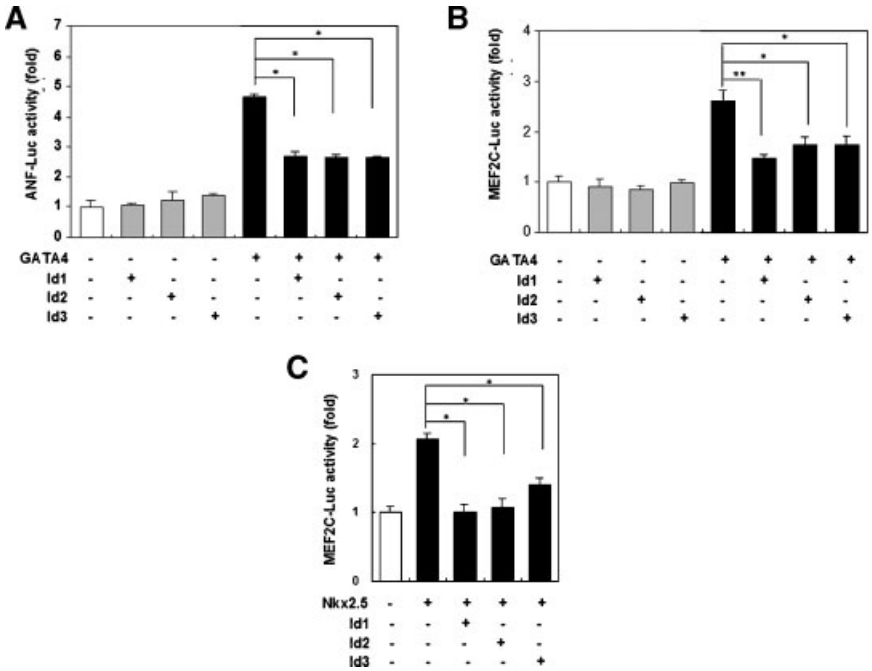


**Fig. 8.** Id proteins suppress the transcriptional activity of GATA4 and Nkx2.5. P19CL6 cells were co-transfected with HA-GATA4 (400 ng/well) and β-galactosidase (50 ng/well) together with either ANF-Luc (**A**) or MEF2C-Luc (**B**) (1 μg/well). After 20 h of incubation, luciferase activity was examined and normalized to β-galactosidase activity. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with HA-GATA4-transfected cells. **C**: Cells were co-transfected with Nkx2.5 (400 ng/well) and β-galactosidase (50 ng/well) together with MEF2C-Luc (1 μg/well). $^{*}P < 0.05$, compared with Nkx2.5-transfected cells.

interact and bind to the Smad/GATA-responsive element within the Nkx2.5 enhancer for the expression of Nkx2.5 [Brown et al., 2004]. Considering several Smad-binding elements are present near the GATA4 and Nkx2.5 binding sites within the Id2 promoter, we cannot exclude the possibility that Smad protein has an effect on GATA4 and Nkx2.5-induced Id2 expression. Nonetheless, two motifs located at −497/−502 and −264/−270 are sufficient for maximal activation of Id2 promoter, serving as binding sites for respective GATA4 and Nkx2.5. Additionally, we could detect that endogenous GATA4 indeed participate in binding to the Id2 promoter region comprising −497/−502 motif in differentiating P19CL6 cells, suggesting this motif is functionally active during differentiation.

In agreement with our model, embryonic localization of Id2 transcript is overlapped with GATA4 and Nkx2.5 expression during heart development. Id2 is also highly expressed in cardiac neural crest, secondary heart field, cardiac outflow/inflow tract, and valves in developing heart, which is similar to cardiac gene expression [Martinsen et al., 2004]. Particularly, Id2 expression in the cranial wall of the distal outflow tract and dorsal mesocardium is consistent with the expressions of GATA4 and Nkx2.5 [Waldo et al., 2001]. Furthermore, a recent effort to identify new Nkx2.5 target genes by microarray analysis in the same P19CL6 cells used as in this study revealed that stable expression of Nkx2.5 increased Id2 mRNA expression [Liu et al., 2005]. These previous reports support our data that GATA4 and Nkx2.5 could play a role as upstream regulators of Id2.

Meanwhile, we observed that overexpression of Ids suppressed GATA4- and Nkx2.5-dependent promoter activation of ANF and MEF2C, which is consistent with the recent study suggesting that Id proteins could function as a negative regulator of GATA4 and Nkx2.5 [Ding et al., 2006]. Ding et al., demonstrated that Id proteins inhibit the transcriptional activity of GATA4 and Nkx2.5, physically interacting with them. They also demonstrated that stable overexpression of Id3 suppressed cardiomyocyte differentiation from P19 cells. Taken together with our study demonstrating GATA4 and Nkx2.5 induce Id2 expression, there seems to

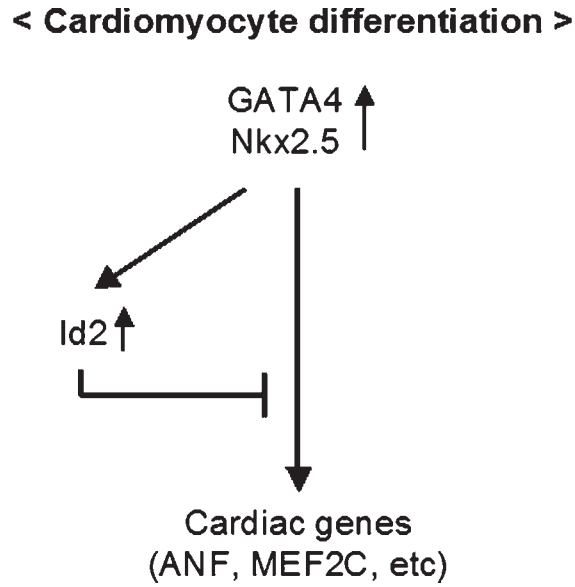


**Fig. 9.** Schematic description of a regulatory relationship between Id2 and cardiac transcription factors GATA4 and Nkx2.5. GATA4 and Nkx2.5 that are induced at early phase of cardiomyocyte differentiation upregulate Id2 expression, and Id2 protein in turn may be involved in fine regulation of the ability of GATA4/Nkx2.5 to activate cardiac gene transcription such as ANF and MEF2C.

be a negative feedback regulatory loop between Id2 and these cardiac transcription factors (Fig. 9). Considering the importance of GATA4 and Nkx2.5 in cardiac development, Id2 induction by GATA4 and Nkx2.5 would be implicated in fine regulation of cardiogenesis at temporal and spatial levels.

In summary, we here demonstrated that Id2 expression is induced by cardiac transcription factors GATA4 and Nkx2.5. Out results suggest that GATA4 and Nkx2.5 could be one of the upstream regulators of Id2.

## REFERENCES

Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. 1990. The Protein Id—A negative regulator of helix-loop-helix DNA-binding proteins. Cell 61:49–59.

Brown CO, Chi X, Garcia-Gras E, Shirai M, Feng XH, Schwartz RJ. 2004. The cardiac determination factor, Nkx2-5, is activated by mutual cofactors GATA-4 and Smad1/4 via a novel upstream enhancer. J Biol Chem 279:10659–10669.

Ding B, Liu CJ, Huang Y, Yu J, Kong W, Lengyel P. 2006. p204 protein overcomes the inhibition of the differentiation of P19 murine embryonal carcinoma cells to beating cardiac myocytes by Id proteins. J Biol Chem 281:14893–14906.

Durocher D, Chen CY, Ardati A, Schwartz RJ, Nemer M. 1996. The atrial natriuretic factor promoter is a downstream target for Nkx-2.5 in the myocardium. Mol Cell Biol 16:4648–4655.

Evans SM, Obrien TX. 1993. Expression of the helix-loop-helix factor Id during mouse embryonic-development. Dev Biol 159:485–499.

Fraidenraich D, Stillwell E, Romero E, Wilkes D, Manova K, Basson CT, Benezra R. 2004. Rescue of cardiac defects in Id knockout embryos by injection of embryonic stem cells. Science 306:247–252.

Gonda H, Sugai M, Nambu Y, Katakai T, Agata Y, Mori KJ, Yokota Y, Shimizu A. 2003. The balance between Pax5 and Id2 activities is the key to AID gene expression. J Exp Med 198:1427–1437.

HabaraOhkubo A. 1996. Differentiation of beating cardiac muscle cells from a derivative of P19 embryonal carcinoma cells. Cell Struct Funct 21:101–110.

Harvey RP. 1996. NK-2 homeobox genes and heart development. Dev Biol 178:203–216.

Heikinheimo M, Scandrett JM, Wilson DB. 1994. Localization of transcription factor Gata-4 to regions of the mouse embryo involved in cardiac development. Dev Biol 164: 361–373.

Hidai C, Masako O, Ikeda H, Nagashima H, Matsuoka R, Quertermous T, Kasanuki H, Kokubun S, Kawana M. 2003. FGF-1 enhanced cardiogenesis in differentiating embryonal carcinoma cell cultures, which was opposite to the effect of FGF-2. J Mol Cell Cardiol 35:421–425.

Hollnagel A, Oehlmann V, Heymer J, Ruther U, Nordheim A. 1999. Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. J Biol Chem 274:19838–19845.

Ip HS, Wilson DB, Heikinheimo M, Tang ZH, Ting CN, Simon MC, Leiden JM, Parmacek MS. 1994. The Gata-4 transcription factor transactivates the cardiac muscle-specific troponin-C promoter-enhancer in nonmuscle cells. Mol Cell Biol 14:7517–7526.

Jen Y, Manova K, Benezra R. 1996. Expression patterns of Id1, Id2, and Id3 are highly related but distinct from that of Id4 during mouse embryogenesis. Dev Dyn 207:235–252.

Karaya K, Mori S, Kimoto H, Shima Y, Tsuji Y, Kurooka H, Akira S, Yokota Y. 2005. Regulation of Id2 expression by CCAAT/enhancer binding protein beta. Nucleic Acids Res 33:1924–1934.

Kasahara H, Usheva A, Ueyama T, Aoki H, Horikoshi N, Izumo S. 2001. Characterization of homo- and heterodimerization of cardiac Csx/Nkx2.5 homeoprotein. J Biol Chem 276:4570–4580.

Kathiriya IS, King IN, Murakami M, Nakagawa M, Astle JM, Gardner KA, Gerard RD, Olson EN, Srivastava D, Nakagawa O. 2004. Hairy-related transcription factors inhibit GATA-dependent cardiac gene expression through a signal-responsive mechanism. J Biol Chem 279:54937–54943.

Kim T, Chen JQ, Sadoshima J, Lee Y. 2004. Jumonji represses atrial natriuretic factor gene expression by inhibiting transcriptional activities of cardiac transcription factors. Mol Cell Biol 24:10151–10160.

Korchynskyi O, ten Dijke P. 2002. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277:4883–4891.

Laverriere AC, Macneill C, Mueller C, Poelmann RE, Burch JBE, Evans T. 1994. Gata-4/5/6, A subfamily of 3 transcription factors transcribed in developing heart and gut. J Biol Chem 269:23177–23184.

Lim JY, Park SJ, Hwang HY, Park EJ, Nam JH, Kim J, Park SI. 2005. TGF-beta 1 induces cardiac hypertrophic responses via PKC-dependent ATF-2 activation. J Mol Cell Cardiol 39:627–636.

Liu H, Harris TM, Kim HH, Childs G. 2005. Cardiac myocyte differentiation: the Nkx2.5 and Cripto target genes in P19 clone 6 cells. Funct Integr Genomics 5:218–239.

Lopez-Rovira T, Chalaux E, Massague J, Rosa JL, Ventura F. 2002. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. J Biol Chem 277:3176–3185.

Lyons I, Parsons LM, Hartley L, Li RL, Andrews JE, Robb L, Harvey RP. 1995. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2–5. Genes Dev 9:1654–1666.

Martinsen BJ, Frasier AJ, Baker CVH, Lohr JL. 2004. Cardiac neural crest ablation alters Id2 gene expression in the developing heart. Dev Biol 272:176–190.

McBride K, Nemer M. 2001. Regulation of the ANF and BNP promoters by GATA factors: Lessons learned for cardiac transcription. Can J Physiol Pharmacol 79:673–681.

Mcburney MW, Jonesvilleneuve EMV, Edwards MKS, Anderson PJ. 1982. Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. Nature 299:165–167.

Molkentin JD, Kalvakolanu DV, Markham BE. 1994. Transcription factor Gata-4 regulates cardiac muscle-specific expression of the alpha-myosin heavy-chain gene. Mol Cell Biol 14:4947–4957.

Molkentin JD, Lin Q, Duncan SA, Olson EN. 1997. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev 11:1061–1072.

Monzen K, Hiroi Y, Kudoh S, Akazawa H, Oka T, Takimoto E, Hayashi D, Hosoda T, Kawabata M, Miyazono K, Ishii S, Yazaki Y, Nagai R, Komuro I. 2001. Smads, TAK1, and their common target ATF-2 play a critical role in cardiomyocyte differentiation. J Cell Biol 153:687–698.

Nakamura T, Sano M, Songyang Z, Schneider MD. 2003. A Wnt- and beta-catenin-dependent pathway for mammalian cardiac myogenesis. Proc Natl Acad Sci USA 100: 5834–5839.

Nakashima K, Takitawa T, Ochiai W, Yanagisawa M, Hisatsune T, Nakafuku M, Miyazono K, Kishimoto T, Kageyama R, Taga T. 2001. BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. Proc Natl Acad Sci USA 98:5868–5873.

Norton JD, Deed RW, Craggs G, Sablitzky F. 1998. Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol 8:58–65.

Ruzinova MB, Benezra R. 2003. Id proteins in development, cell cycle and cancer. Trends Cell Biol 13:410–418.

Simon MC. 1995. Gotta have GATA. Nat Genet 11:9–11.

Springhorn JP, Ellingsen O, Berger HJ, Kelly RA, Smith TW. 1992. Transcriptional regulation in cardiac-muscle—Coordinate expression of Id with a neonatal phenotype during development and following a hypertrophic stimulus in adult-rat ventricular myocytes in vitro. J Biol Chem 267:14360–14365.

Wadman IA, Osada H, Grutz GG, Agulnick AD, Westphal H, Forster A, Rabbitts TH. 1997. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-bindinq complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. EMBO J 16:3145–3157.

Waldo KL, Kumiski DH, Wallis KT, Stadt HA, Hutson MR, Platt DH, Kirby ML. 2001. Conotruncal myocardium arises from a secondary heart field. Development 128: 3179–3188.