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Two Potent Transactivation Domains in the C-Terminal Region of Human NANOG Mediate Transcriptional Activation in Human Embryonic Carcinoma Cells

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ABSTRACT

The core embryonic stem cell transcription factors Oct4, Sox2, and Nanog are expressed in germ cell tumors (GCTs) and have been proposed to play a regulatory role in tumorigenesis. However, little is known about the mechanism of regulation of tumorigenesis by the complicated network of these proteins. Nanog is a novel homeobox-containing transcription factor that is expressed in pluripotent cells as well as GCTs. To understand the molecular and functional role of human NANOG (hNANOG) in germ cells, mutagenesis of the C-terminal domain (CD) of hNANOG and transient transfection assays in NCCIT human embryonic carcinoma cells were carried out to identify critical transactivation motifs. We divided the CD into three putative functional subdomains, CD1, tryptophan-repeat (WR) subdomain, and CD2. WR subdomain and CD2 independently contained transcriptional potential and, in combination, had a synergistic effect on transcriptional activity, while CD1 was transcriptionally inactive. The glutamine (Q) motif in WR subdomain, and multiple acidic residues in CD2 were required for maximal and synergistic transcriptional activation by the hNANOG CD. The results of the current study contribute to a better understanding of the complicated molecular machinery of stem cell transcription factors and their role in unregulated proliferation in germ cell tumorigenesis. J. Cell. Biochem. 106: 1079–1089, 2009.

KEY WORDS: HUMAN NANOG; TRANSACTIVATION DOMAIN; TRYPTOPHAN-REPEAT; GLUTAMINE; ACIDIC RESIDUES; HUMAN EMBRYONIC CARCINOMA; NCCIT

In recent years, stem cells have been the focus of considerable interest because of their potential to serve as tools for understanding early development, as well as their therapeutic potential in regenerative medicine. Multiple, complex transcriptional regulatory networks appear to be involved in the maintenance of embryonic stem cell (ESC) pluripotency and self-renewal [Pera and Trounson, 2004; Boiani and Scholer, 2005; Boyer et al., 2005]. Among the regulatory factors and signaling molecules of interest, several transcription factors, including Oct4, Sox2, and Nanog, are thought to play central roles in the maintenance of stemness through co-occupancy of promoters of target genes that are specific to stemness or differentiation [Boyer et al., 2005]. Oct4/Sox2 heterodimers regulate Nanog expression through binding to octamer/sox elements within the Nanog proximal promoter region, which suggests that the Oct4/Sox2 complex may be a key regulator of pluripotency [Kuroda et al., 2005; Rodda et al., 2005].

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Oct4, Sox2, and Nanog are also expressed in human GCTs, suggesting a crucial role for transcription factor networks in the regulation of GCT growth and differentiation [Clark, 2007; Santagata et al., 2007]. Testicular GCTs can proliferate into seminomas or develop into non-seminomas, depending on the cell type and degree of differentiation [Kristensen et al., 2008]. Seminomas resemble transformed primordial germ cells and exhibit a similar gene expression profile to ESCs. Non-seminoma GCTs can be divided into undifferentiated embryonal carcinomas, differentiated choriocarcinomas, and yolk sac tumors. Extensive immunohistochemical analysis has shown that Nanog and Oct4 are sensitive and specific markers for primary seminomas and embryonal carcinomas [Jones et al., 2004]. Sox2 expression is restricted to embryonal carcinomas, and is not detected in pure seminomas, which suggests that Sox2 may be a useful diagnostic marker for distinguishing seminomas and non-seminomas [Santagata et al., 2007].

Nanog, a novel homeobox-containing transcription factor, is a key regulator of ESC self-renewal and pluripotency [Mitsui et al., 2003]. Nanog-deficient embryonic stem (ES) cells and embryos lose their pluripotency. Moreover, Nanog overexpression leads to the clonal expansion of ES cells through circumvention of the leukemia inhibitory factor (LIF)-dependent Stat3 pathway and sustained Oct4 expression levels [Chambers et al., 2003; Mitsui et al., 2003]. Genome-wide gene expression profiling shows that Nanog is expressed at high levels in testicular carcinoma in situ and GCTs. Nanog is also strongly expressed in fetal gonocytes, and is down-regulated earlier than Oct4, which suggests that Nanog functions as a regulatory factor upstream of Oct4 in germ cells [Hoei-Hansen et al., 2005]. Recently, it was reported that Nanog activates Oct4 promoter transcriptional activity in the mouse [Pan et al., 2006]. However, the molecular mechanism of regulation of germ cell tumorigenesis by these stem cell transcription factors remains largely unknown. Recently, in efforts to understand the function of Nanog in the transcriptional regulation of downstream target genes, a potent transactivation domain was identified in murine Nanog (mNanog) and hNANOG [Pan and Pei, 2003; Oh et al., 2005]. mNanog contains transcriptional activity in regions both N- and C-terminal to the homeodomain (HD), and two potent transcriptional activation subdomains are present in the C-terminus of mNanog [Pan and Pei, 2003, 2005]. We previously reported that hNANOG possesses transcriptional activity only in its C-terminus [Oh et al., 2005]. Here, we divided the CD of hNANOG into three subdomains, CD1, WR (tryptophan-repeat) subdomain, and CD2. These subdomains, individually and in combination, were expressed in NCCIT cells, which have an intermediate phenotype between seminoma and embryonal carcinoma cells and are a useful tool for studying the relationship between seminoma and non-seminoma tumorigenesis [Damjanov et al., 1993]. We demonstrated that two potent transactivation subdomains are present in the CD of hNANOG, WR subdomain and CD2, which synergistically activate transcriptional activity in NCCIT, human embryonic carcinoma (hEC) cells. We identified that Q residues in WR subdomain and an acidic transactivation motif within CD2 are critical for transactivation activity. We also demonstrated that both of these motifs, in WR subdomain and CD2, are required for maximal and synergistic transcriptional activity by hNANOG in hEC cells.

MATERIALS AND METHODS

PLASMID CONSTRUCTS

The hNANOG deletion and amino acid substitution constructs were generated by the "QuickChange" method (Stratagene, La Jolla, CA) using full-length hNANOG cDNA isolated from NCCIT cells as a template. To construct fusion proteins with the GAL4 DNA binding domain (GAL4 DBD; amino acids 1–147), sequences corresponding to hNANOG CD wild-type (WT) and CD deletion or substitution mutants were inserted into the multiple cloning site of pBIND (Promega, Madison, WI), and then fused in-frame to the C-terminus of GAL4 DBD. Expression vectors for full-length hNANOG WT and its mutant derivatives tagged with the Flag epitope were constructed by inserting the corresponding sequences into pcDNA3.1. All PCR reactions were performed using pfu DNA polymerase (Core Bio System, Seoul, Republic of Korea). A minimal promoter construct, pGL3ti [Jonk et al., 1998], was constructed by inserting the adenovirus major late promoter TATA box and the mouse terminal deoxynucleotidyl transferase gene initiator sequence into the multiple cloning site of pGL3-basic (Promega). A reporter construct bearing the Nanog binding site (pGL3ti-Nanog BS, Fig. 6A) was constructed by inserting an oligonucleotide containing three copies of the Nanog binding site [ACCCTTCGCCGATTAAGTACTTAA; Pan and Pei, 2005] upstream of the minimal promoter of pGL3ti. All cloned PCR products and reporter plasmids were verified by sequencing.

CELL CULTURE

NCCIT, HEK293T (human embryonic kidney), and HeLa (human cervical cancer) cells (American Type Cell Collection) were grown in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 IU penicillin, and 100 μg/ml streptomycin.

REPORTER ASSAYS

Cells were cotransfected with 0.5 μg pG5*luc* (Promega), which contained five copies of the GAL4 DNA binding site, or pGL3ti-Nanog BS, and 1 μg each of the appropriate GAL4 DBD- or Flag-tagged protein expression vector using the ExGen500 in vitro transfection reagent (Fermentas, Hanover, MD) in 6-well tissue culture plates. Cells were cotransfected with an internal control plasmid, pcDNA3.1/hygro/*Lac*Z (Invitrogen, Carlsbad, CA), and the luciferase activities of the reporter plasmids were normalized to β-galactosidase activity. Cells were harvested 48 h after transfection and luciferase activity was measured using the firefly luciferase assay system (Promega). Transfection and reporter assay experiments were carried out in duplicate and independently repeated more than three times.

WESTERN BLOT ANALYSIS

HEK293T cells were transfected with expression vectors for GAL4 DBD- or Flag-tagged proteins of hNANOG using the ExGen500 in vitro transfection reagent, according to the manufacturer's protocol,

and harvested 48 h after transfection. For Western blot analysis, cells were lysed with 100 μl of Pro-PREP protein extraction solution (iNtRON Biotech., Inc., Sungnam, Republic of Korea) on ice for 20 min. Whole cell lysate was separated by 10–12% SDS–polyacrylamide gel electrophoresis and proteins were transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in 5% non-fat milk, and then incubated with an anti-GAL4 monoclonal antibody (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-Flag monoclonal antibody (1:20,000, Sigma, St. Louis, MO), followed by incubation with a horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5,000, Santa Cruz). Immunoreactive proteins were visualized using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Flag-tagged hNANOG WT and mutant proteins were synthesized using the TNT Quick Coupled Transcription/Translation System (Promega), according to the manufacturer's protocol, and expression was confirmed by Western blot analysis using an anti-Flag antibody (Sigma), as described above.

Binding activity assays were performed as described previously [Yang et al., 2005, 2007]. Briefly, oligonucleotides containing the Nanog binding element were annealed and radiolabeled. In each binding reaction, 3 μl of in vitro translation product was preincubated in a total volume of 15 μl in 50 mM Tris–HCl (pH 7.4), 0.5% Triton X-100, 5 mM EDTA, 20% glycerol, 1 μg poly(dG:dC), 50 mM 2-β-mercaptoethanol, and 0.5 μg BSA on ice for 10 min. Samples were then incubated with 1 μl of radiolabeled probe (20,000 cpm) on ice for 15 min. DNA–protein complexes were separated from free probe by native 4% polyacrylamide gel electrophoresis, and then visualized by autoradiography. The oligonucleotide sequences of the EMSA probes were 5′-ACCCTTCGCCGATTAAGTACTTAA-3′ and ACCCTTCGCCGAcgAAGTACcTAA, for the WT and mutant probe, respectively (mutated nucleotides in lower case).

RESULTS

MAPPING THE TRANSACTIVATION DOMAIN IN THE C-TERMINAL REGION OF hNANOG

Previously, we reported that the transcriptional activity of hNANOG localizes to the CD [Oh et al., 2005]. To characterize the transactivation domain of hNANOG in more detail, we divided the CD into three putative functional subdomains, CD1 [amino acids (aa) 155–195], WR subdomain (aa 196–239), and CD2 (aa 240–305), based on the corresponding regions of mNanog [Pan and Pei, 2005] (Fig. 1).

CD1, WR subdomain and CD2, either individually or in various combinations, were fused to GAL4 DBD, which alone lacks the ability to stimulate transcription (Fig. 2A). Expression of the subdomains was confirmed by Western blot using an anti-GAL4 DBD monoclonal antibody (Fig. 2B). To determine the transcriptional potential of individual subdomains, we performed transient transfection assays in undifferentiated NCCIT hEC cells and differentiated HEK293T cells. Cells were transiently cotransfected with a reporter construct, pG5*luc*, which contained five copies of the GAL4 DNA binding site upstream of a minimal promoter, and

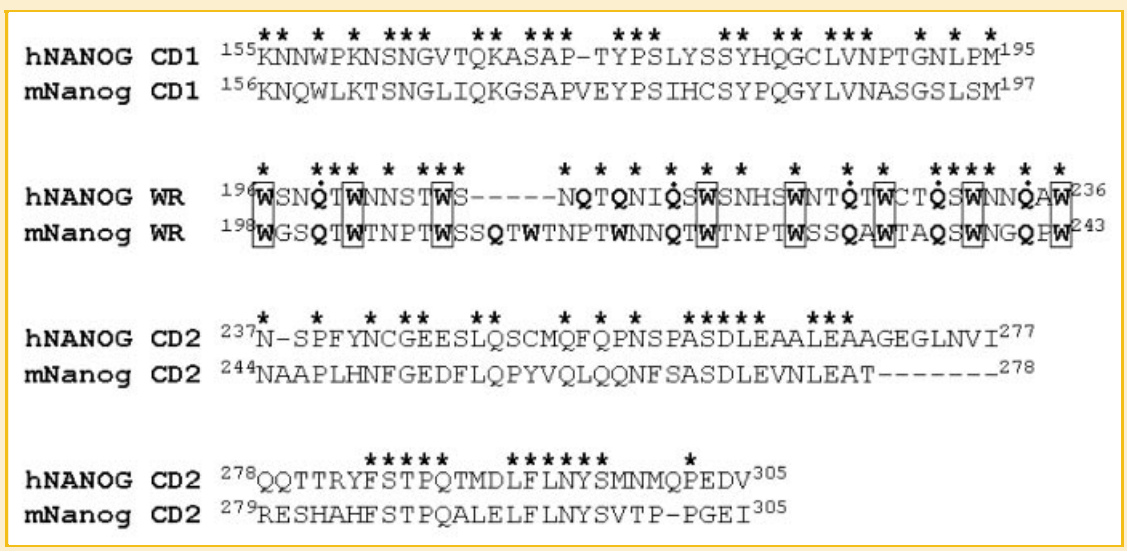

Fig. 1. Alignment of human and murine Nanog amino acid sequences. The hNANOG CD was divided into three subdomains, corresponding to analogous regions of mNanog. CD1, WR subdomain, and CD2 of hNANOG are shown in the upper rows, and the respective subdomains of mNanog are shown in the lower rows. The numbers above each diagram represent amino acid sequences and asterisks indicate identical residues. W and Q residues within WR subdomain are in bold. Within WR subdomain, squares and dots represent the eight W and five Q residues, respectively, which are conserved between the two species. The amino acid sequence of hNANOG is approximately 52% identical to mNanog in the full-length CD and 56% identical in the WR subdomain.

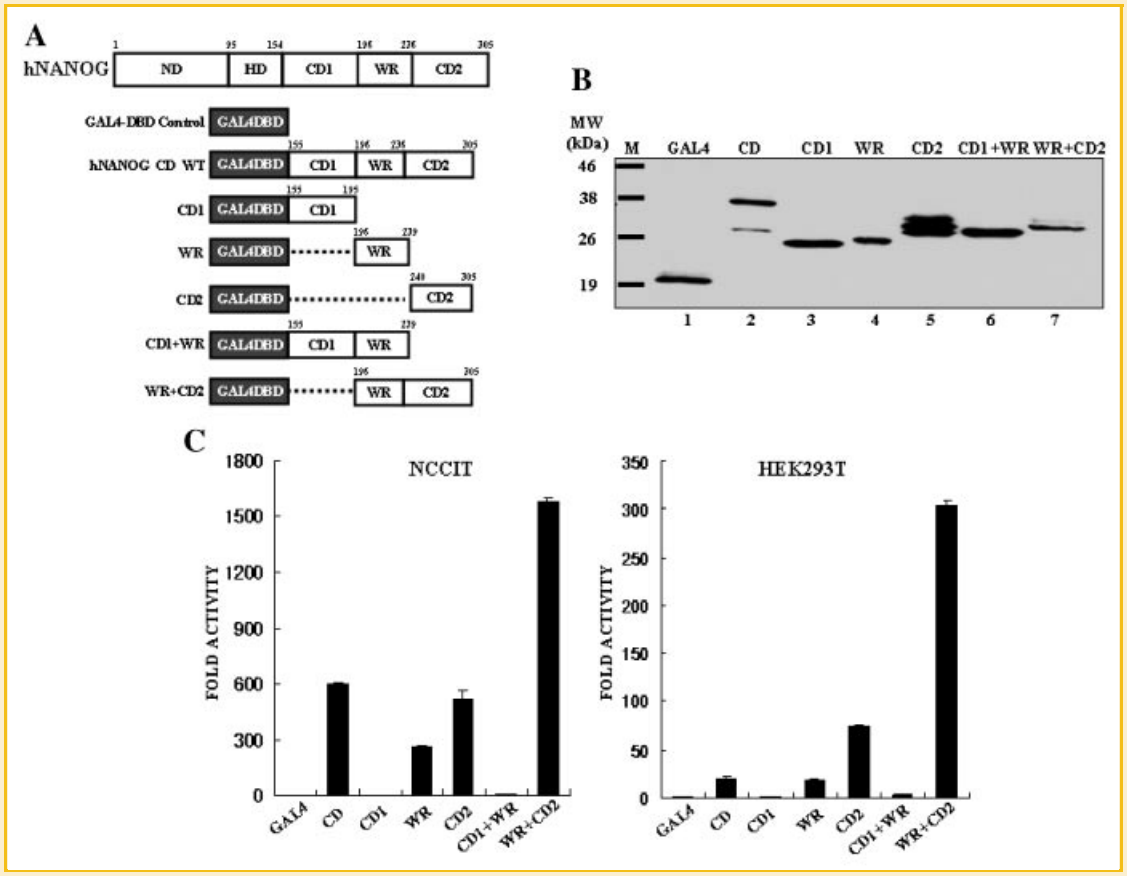

Fig. 2. Mapping of the transactivation domains of the hNANOG CD. A: Schematic representation of full-length and C-terminal truncation mutants of hNANOG CD fused to GAL4 DBD. ND, N-terminal domain; HD, homeodomain. B: Western blot analysis of HEK293T cells transfected with GAL4 DBD fusion constructs using an anti-GAL4 antibody. The lanes are as follows: control (lane 1), hNANOG CD WT (lane 2), CD1 (lane 3), WR subdomain (lane 4), CD2 (lane 5), CD1 + WR subdomain (lane 6), and WR subdomain + CD2 (lane 7). C: Transcriptional activity of the indicated constructs in NCCIT and HEK293T cells. Activity was normalized to that of the reporter gene, pG5*luc* alone, which was set as 1. Data represent the means and standard deviation (SD) of duplicate measurements from one representative transfection.

luciferase activity was measured, as described previously [Oh et al., 2005]. WR subdomain or CD2, in the absence of CD1, independently and significantly induced luciferase activity in both NCCIT and HEK293T cells (Fig. 2C). Nevertheless, there were some differences in the levels of activation. In NCCIT cells, fold-activation by either WR subdomain or CD2 alone was lower than that of the full-length CD, while in HEK293T cells, CD2 alone significantly increased the transcriptional activity of the reporter gene, compared to that of the full-length CD. The combination of WR subdomain and CD2 dramatically activated reporter gene expression as compared to full-length CD, WR subdomain or CD2 alone in both cell lines. Neither CD1 alone nor the combination of CD1 and WR subdomain exhibited significant activity. Thus, both WR subdomain and CD2 of hNANOG were required for maximum transcriptional activity.

THE Q-MOTIF WITHIN WR SUBDOMAIN IS CRUCIAL FOR FULL TRANSCRIPTIONAL ACTIVITY OF THE hNANOG CD

The WR is an important structural and functional motif for DNA binding, transcriptional activity and dimerization [Saikumar et al., 1990; Pan and Pei, 2005; Mullin et al., 2008; Wang et al., 2008]. To determine the contribution of the WR within WR subdomain to transcriptional activity, we carried out deletion and site-directed mutagenesis of WR subdomain in the full-length CD, and examined the transcriptional activity of the mutants using the GAL4 DBD reporter/fusion protein system (Fig. 3A,B). Expression of WR subdomain mutants was confirmed by Western blot analysis (Fig. 3C). Deletion of WR subdomain (aa 196–236) increased the transcriptional activity of the CD significantly in NCCIT and HEK293T cells. Substitution of eight W residues in the WR resulted in a slight decrease in the transcriptional activity of the CD in NCCIT cells as compared to hNANOG CD WT, but increased activity approximately threefold in HEK293T cells (Fig. 3D). These results suggest that WR subdomain within the CD may be tightly regulated by as-yet unknown factor(s), and/or that the WR be dispensable for the transcriptional activity of the hNANOG CD in NCCIT cells.

To identify the motif(s) within WR subdomain that were critical for transcriptional activity, we carried out site-directed mutagenesis of seven Q residues in WR subdomain that were previously shown to be an important feature of the transactivation domains of other

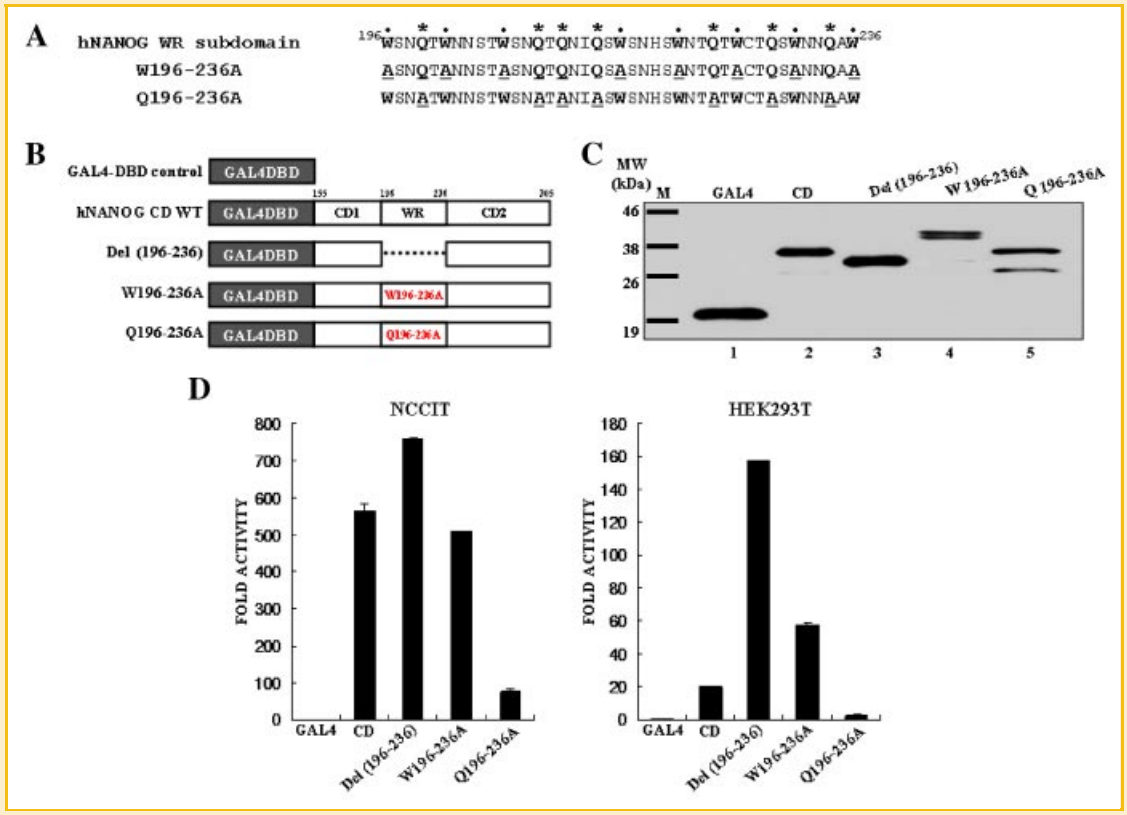

Fig. 3. A Q-rich motif, not the WR, within WR subdomain is crucial for full transcriptional activity of the hNANOG CD. A: Amino acid sequences of hNANOG WR subdomain and subdomain mutants (W196–236A and Q196–236A). Asterisks or dots indicate the seven Q residues or eight W residues, respectively, of WR subdomain. Substituted W or Q residues and the A replacements are shown in bold and underlined, respectively. B: Schematic representation of WR subdomain mutants of hNANOG CD fused to GAL4 DBD. The constructs are as follows: CD WT, full-length CD (aa 155–305); CD-Del (196–236), deletion of aa 196–236; CD (W196–236A), mutation of W residues to A within aa 196–236; and CD (Q196–236A), mutation of Q residues to A within aa 196–236. C: Western blot analysis of HEK293T cells transfected with GAL4 DBD fusion constructs using an anti-GAL4 antibody. The lanes are as follows: control (lane 1), hNANOG CD (lane 2), Del (196–236) (lane 3), W196–236A (lane 4), Q196–236A (lane 5). D: Transcriptional activity of the indicated constructs in NCCIT and HEK293T cells was calculated relative to the expression of pG5/*luc* alone, which was set as 1. Data represent the means and SD of duplicate measurements from one representative transfection.

transcription factors [Tanaka et al., 1994; Klemsz and Maki, 1996; Stepchenko and Nirenberg, 2004] (Fig. 3A,B). Sequence alignment showed that of the seven Q residues in hNANOG WR subdomain, five residues are well conserved in mNanog (Fig. 1). Expression of the Q substitution mutant was confirmed by Western blot analysis (Fig. 3C, lane 5). Mutation of WR subdomain Q residues to alanine (A) dramatically decreased the transcriptional activity of the CD by approximately 90%, even in the presence of CD2 (Fig. 3D). These results suggested that the Q residues of WR subdomain are an essential transactivation motif, and are required for the full transcriptional activity of hNANOG.

ACIDIC MOTIFS WITHIN AA 253–273 ARE REQUIRED FOR CD2 TRANSCRIPTIONAL ACTIVITY

To identify the functional motifs that were involved in the transactivation activity of CD2, we generated a series of CD2 deletion mutants in the context of full-length CD (CD WT) (Fig. 4A). Expression of the deletion mutants was confirmed by Western blot (Fig. 4B). The transcriptional activity of CD (155–286) was decreased by less than 20% as compared to CD WT, which suggested that this motif is of limited importance in the regulation of hNANOG transcriptional activity. CD (155–252) exhibited a dramatic decrease in transcriptional activity, indicating the presence of a crucial transactivation motif within aa 253–286 of CD2. Deletion of CD2 resulted in a further decrease in transcriptional activity, even in the presence of intact WR subdomain. These results suggested that full-length CD2 is required for full transcriptional activity of the hNANOG CD. In-frame deletion of aa 253–273 completely abolished the transcriptional activity of the CD, even in the presence of intact WR subdomain. These results indicated that aa 253–273 of CD2 are required for full transcriptional activity of hNANOG CD (Fig. 4C).

Acidic motifs play a crucial role in the transactivation activity of several transcription factors [Seipel et al., 1992]. To determine whether acidic residues within aa 253–273 of CD2 were important for transcriptional activity, we used site-directed mutagenesis to change single and multiple glutamate (E) residues in this region to A or glycine (G) (Fig. 5A). Expression of mutant constructs was

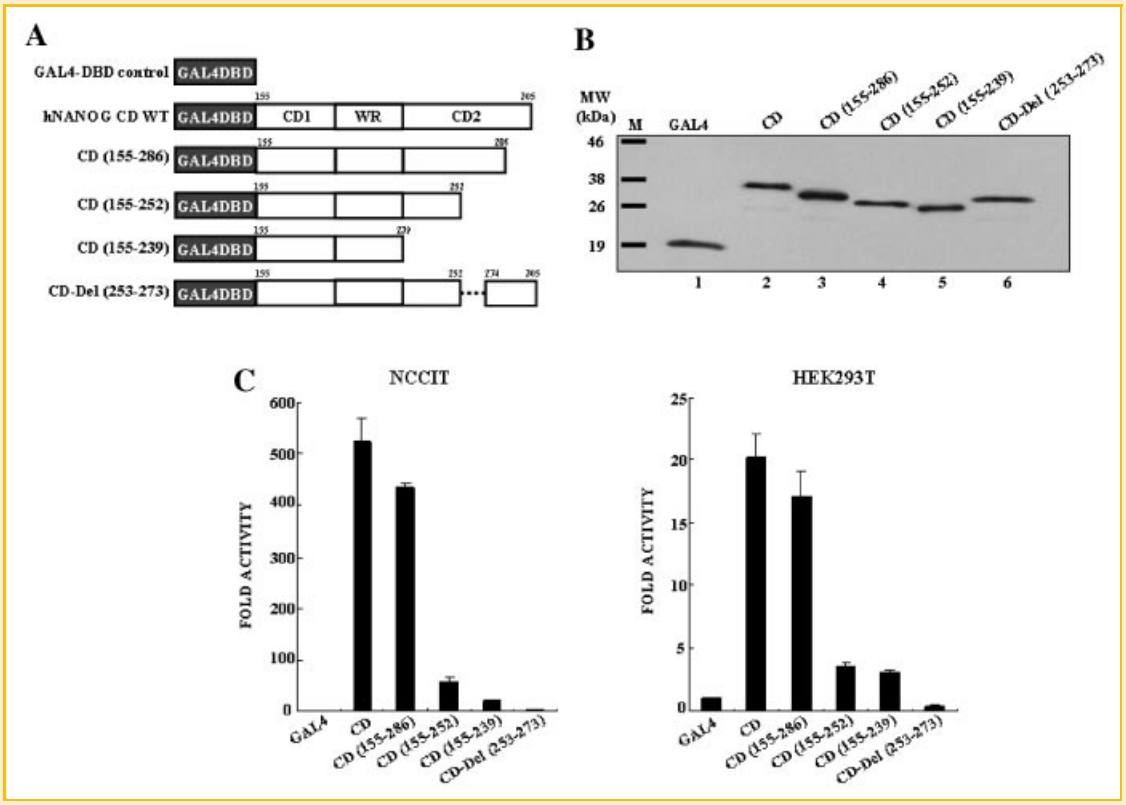

Fig. 4. CD2 transcriptional activity requires aa 253–273. A: Schematic representation of hNANOG CD2 deletion mutants fused to GAL4 DBD. The numbers above each diagram represent the amino acid sequences of hNANOG CD. B: Western blot analysis of HEK293T cells transfected with GAL4 DBD fusion constructs using an anti-GAL4 antibody. The lanes are as follows: control (lane 1), hNANOG CD WT (lane 2), CD (155–286) (lane 3), CD (155–252) (lane 4), CD (155–239) (lane 5). CD-Del (253–273) (lane 6). C: Transcriptional activity of the indicated constructs in NCCIT and HEK293T cells was calculated relative to the expression of pG5*luc* alone, which was set as 1. Data represent the means and SD of duplicate measurements from one representative transfection.

confirmed by Western blot analysis (Fig. 5B). In both NCCIT and HEK293T cells, single substitution of individual E residues decreased the transcriptional activity of the CD to a similar extent, whereas multiple substitutions of two or three E residues resulted in a significant and dramatic decrease in activity (Fig. 5C). These data indicated that acidic residues within aa 253–273 are crucial for the transcriptional activity of CD2. Our results provide strong evidence that both the Q-motif in WR subdomain and multiple E residues in CD2 are required for maximal transcriptional activity of hNANOG.

ROLE OF Q AND ACIDIC RESIDUES IN TRANSACTIVATION BY FULL-LENGTH hNANOG

To confirm the transcriptional potential of WR subdomain and CD2 in the context of full-length hNANOG, we generated a luciferase reporter construct that contained three copies of the Nanog binding site (pGL3ti-Nanog BS). We also constructed expression vectors for Flag-tagged deletion and site-directed mutants of full-length hNANOG (Fig. 6A). Western blot analysis using an anti-Flag monoclonal antibody confirmed the expression of full-length hNANOG and its mutant derivatives (Fig. 6B). We previously reported that the nuclear localization signal of hNANOG is restricted to the homeobox DNA binding domain [Do et al., 2007]. Thus, we were able to rule out the possibility that any changes in the transcriptional activity of hNANOG induced by mutation were due to impaired nuclear localization.

To evaluate the transcriptional activity of the hNANOG deletion and substitution mutants, HEK293T and HeLa cells were cotransfected with pGL3ti-Nanog BS and expression vectors for hNANOG WT or mutants. NCCIT cells were not suitable for these assays due to competition for binding to the reporter gene by endogenous hNANOG. As seen in Figure 6C, substitution of W residues to A in WR subdomain significantly increased the transcriptional activity of full-length hNANOG, while mutation of Q residues to A resulted in a dramatic decrease in transcriptional activity. Thus, Q residues in WR subdomain were a critical component of transactivation, whereas the WR appeared to be dispensable in the context of full-length hNANOG. Multiple substitutions of acidic E residues in CD2 resulted in a dramatic decrease (>85% as compared to WT) in the transcriptional activity of full-length hNANOG. These results indicated that Q residues in WR subdomain and multiple acidic E residues in CD2 function in a constitutive and synergistic manner as potent transactivation motifs of hNANOG. To confirm the

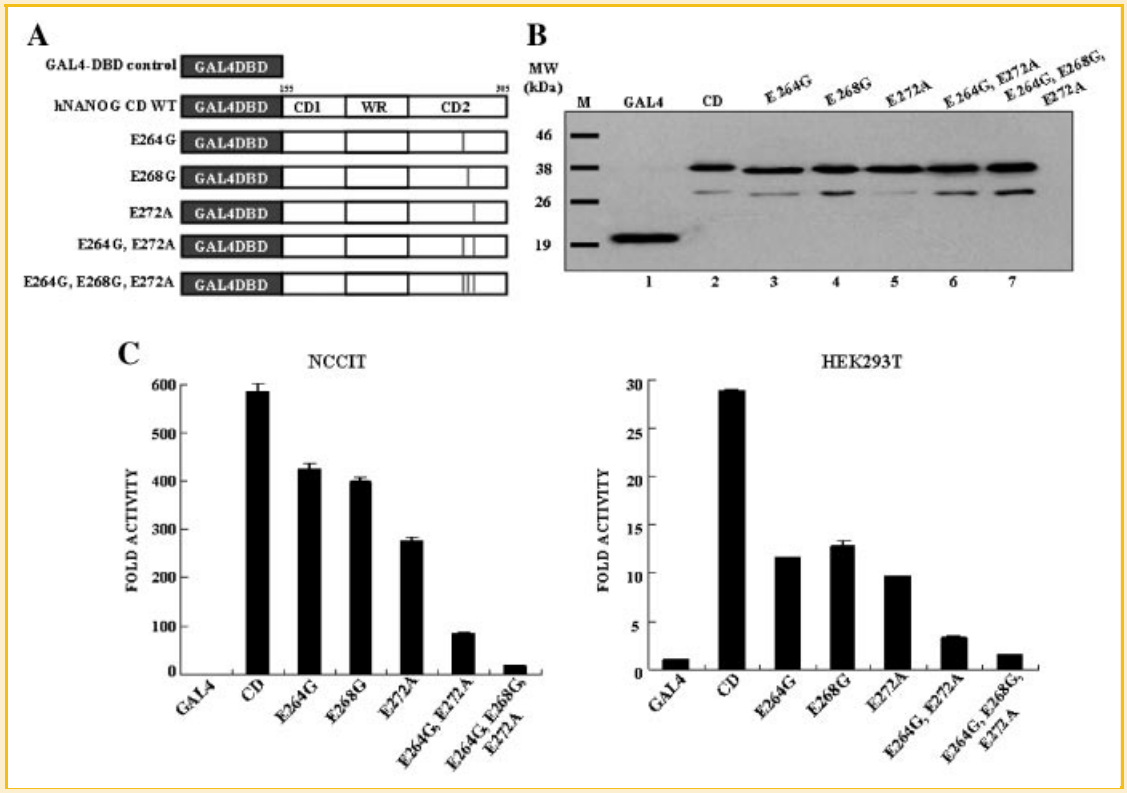

Fig. 5. Acidic residues within aa 253–273 are required for CD2 transcriptional activity. A: Schematic representation of hNANOG CD site-directed mutants fused to GAL4 DBD. B: Western blot analysis of HEK293T cells transfected with GAL4 DBD fusion constructs using an anti-GAL4 antibody. The lanes are as follows: control (lane 1), hNANOG CD WT (aa 155–305) (lane 2), CD (E264G) (lane 3), CD (E268G) (lane 4), CD (E272A) (lane 5), CD (E264G, E272A) (lane 6), and CD (E264G, E268G, E272A) (lane 7). C: Transcriptional activity of the indicated constructs in NCCIT and HEK293T cells was calculated relative to the expression of pG5*luc* alone, which was set as 1. Data represent the means and SD of duplicate measurements from one representative transfection.

importance of these motifs, we generated a mutant of full-length hNANOG in which critical Q and E residues in WR subdomain and CD2, respectively were mutated (Fig. 7A). Expression of this mutant was confirmed by Western blot analysis (Fig. 7B). Mutation of Q and E residues in WR subdomain and CD2 resulted in a significant decrease in transcriptional activity as compared to hNANOG WT (Fig. 7C).

To confirm that the loss of transcriptional activity of full-length hNANOG mutants was not due to changes in DNA binding activity, we performed EMSAs of hNANOG deletion and substitution mutants (WR subdomain and aa 253–273 deletion mutants, W and Q substitution mutants of WR domain, and E substitution mutants of aa 253–273). Expression of Flag-tagged proteins was confirmed by Western blot analysis (Fig. 8A). EMSA showed that all of the hNANOG mutants, as well as hNANOG WT, retained their binding activity, indicating that binding was independent of transcriptional activity (Fig. 8B). The binding specificity of WT and mutant proteins was also analyzed using a 50-fold molar excess of unlabeled Nanog WT and mutant (MT) probes. The binding activity of full-length hNANOG WT and mutants decreased upon co-incubation with unlabeled WT probe and was unaffected by incubation with MT probe. These results indicated that the impaired transcriptional activity of hNANOG mutants was not due to changes in binding activity, but rather, to changes in WR subdomain and CD2 that effected transactivation activity.

DISCUSSION

Nanog was first identified in a screen for pluripotency-promoting genes, and its expression was detected in human GCTs as well as pluripotent ESCs [Chambers et al., 2003; Mitsui et al., 2003; Clark et al., 2004; Hart et al., 2004, 2005]. The Nanog gene was mapped to human chromosome 12p13, which is known to be a hot spot for germ cell tumorigenesis [Clark et al., 2004]. It has been proposed that the molecular machinery that regulates stem cell self-renewal is reactivated in the dysregulated proliferation of tumorigenesis [Hart et al., 2004]. Nevertheless, little is known about the regulatory mechanism of stem cell transcription factors in GCTs. In an effort to understand the functional role of Nanog as a transcriptional regulator in GCTs, we obtained the full-length hNANOG cDNA from NCCIT cells, which strongly express hNANOG, and analyzed transcriptional activity in pluripotent NCCIT cells and non-pluripotent HEK293T and HeLa cells.

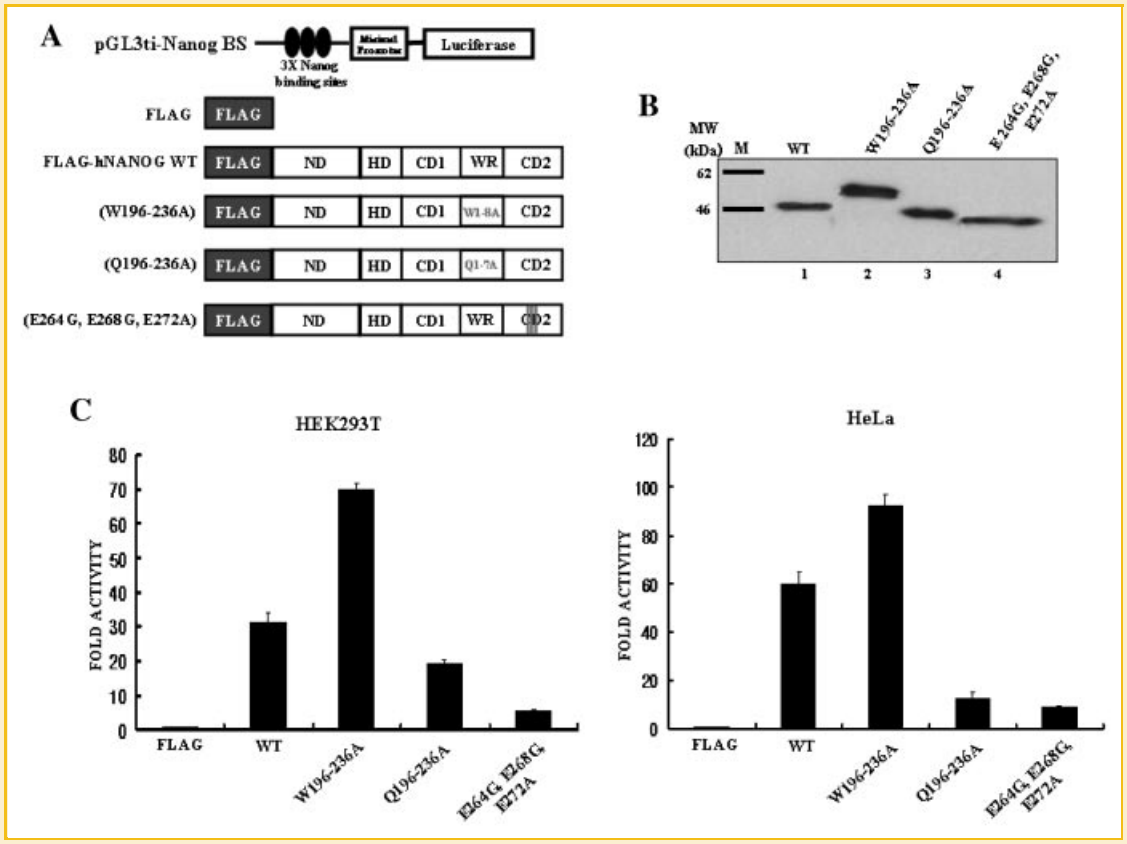

Fig. 6. Function of Q-motif and acidic E residues in transcriptional activation by full-length hNANOG. A: Schematic representation of full-length hNANOG deletion and site-directed mutant derivatives tagged with the Flag epitope. B: Western blot analysis HEK293T cells transfected with Flag-tagged constructs using an anti-Flag antibody. The lanes are as follows: full-length hNANOG WT (lane 1), W196–236A (lane 2), Q196–236A (lane 3), (E264G, E268G, E272A) (lane 4). C: Transcriptional activity of the indicated constructs in HEK293T and HeLa cells was calculated relative to the expression of the reporter gene, pGL3ti-Nanog BS, which was set as 1. Data represent the means and SD of duplicate measurements from one representative transfection.

While mNanog contains transcriptional activation potential in two regions, the N-terminal domain (ND) and CD, the CD possesses a much higher activation potential than the ND [Pan and Pei, 2003]. Previously, we found that the transcriptional activity of hNANOG maps to one region, the CD [Oh et al., 2005]. Amino acid sequence alignment showed that hNANOG shares only 58% identity over the full length sequence with mNanog, and 87% identity in the HD [Chambers et al., 2003], which suggests that the functional motifs of Nanog may differ between the two species. The amino acid identity between the two proteins is even lower in the CD (52% identity) and WR subdomain (56% identity). There are ten W residues in WR subdomain of mNanog and eight in hNANOG, all of which are conserved in mNanog. To identify the functional motifs of hNANOG that are critical for transcriptional activity, we divided the CD into three putative functional regions, CD1 (aa 155–195), WR subdomain (aa 196–239), and CD2 (aa 240–305), based on WR subdomain sequences of hNANOG (Fig. 1).

We identified two potent transactivation domains in hNANOG (WR subdomain and CD2) that significantly and synergistically activated luciferase reporter gene transcription in pluripotent hEC cells (Fig. 2). Multiple acidic E residues in CD2 and the Q-motif in WR subdomain were crucial for transcriptional activity. We also demonstrated that both of these transactivation motifs are required for maximal and synergistic transcriptional activity of hNANOG in pluripotent NCCIT cells (Figs. 3, 5, and 6). Furthermore, these two potent transactivation domains in the CD of hNANOG had different transactivation properties, which suggest that distinct regulatory factors and pathways might be involved in the dysregulation of proliferation in germ cell tumorigenesis, and in the maintenance of stem cell characteristics.

WR subdomain of hNANOG contains 7 Q residues (17% of WR subdomain), 13 serine/threonine residues (S/T, 32%), and 9 asparagines (N, 22%), as well as 8 W residues (20%). In mNanog, the W-motif is required for transcriptional activity, while other residues do not appear to be involved. However, deletion of WR subdomain of full-length mNanog strongly induces transcriptional activity, which suggests that this domain is dispensable for the transcriptional activity of full-length mNanog [Pan and Pei, 2005]. In the current study, mutation of W residues in WR subdomain significantly increased the transcriptional activity of full-length hNANOG (Fig. 6), which was in agreement with previous results. However, Q residues of WR subdomain were more critical for the

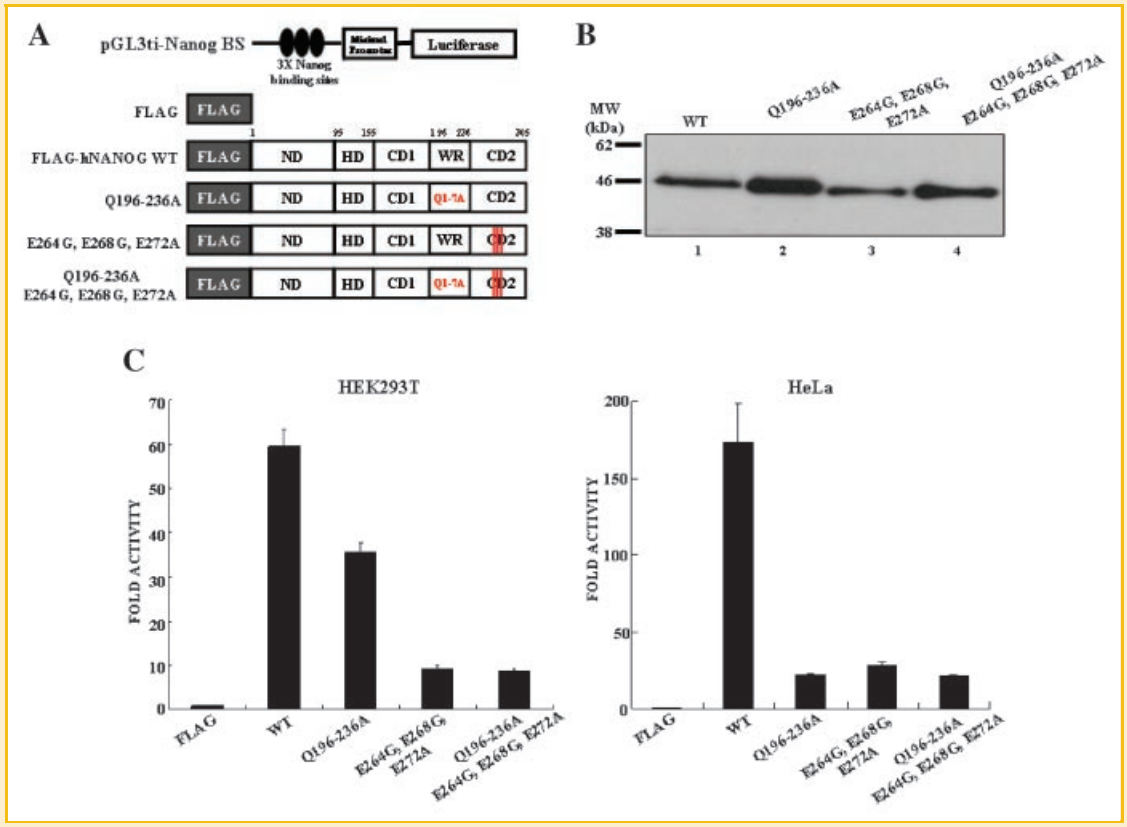

Fig. 7. Both Q-motif and multiple acidic E residues are required for full transcriptional activity of hNANOG. A: Schematic representation of full-length hNANOG and its deletion and site-directed mutant derivatives fused to the Flag epitope. B: Western blot analysis of HEK293T cells transfected with Flag-tagged constructs using an anti-Flag antibody. Lanes are as follows: WT (lane 1); Q196–236A (lane 2); E264G, E268G, E272A (lane 3); Q196–236A, E264G, E268G, E272A (lane 4). C: Transcriptional activity of the indicated constructs in HEK293T and HeLa cells was calculated relative to the expression of pGL3ti-Nanog BS alone, which was set as 1. Data represent the means and SD of duplicate measurements from one representative transfection.

transcriptional activity of hNANOG, which indicates that hNANOG has different transactivation motifs than mNanog. Western blot analysis of GAL4 DBD fusion proteins revealed that the W substitution mutants migrated to higher positions and generated multiple bands (Figs. 3C and 6B), similar to mNanog [Pan and Pei, 2005]. These results suggest that substitution of the W residues of WR subdomain result in structural changes in hNANOG. The WR of the c-myb protooncogene forms a hydrophobic scaffold that maintains the structural integrity of the DNA-binding domain [Saikumar et al., 1990]. Recent studies have shown that the WR of mNanog is involved in mNanog dimerization. In particular, W residues play a crucial role in dimerization and the maintenance of LIF-independent self-renewal in mouse ES cells [Mullin et al., 2008; Wang et al., 2008]. Additional studies are needed to elucidate the mechanism by which the WR regulates the transactivation activity of Nanog. A second region of the CD, CD2, also exhibited significant transcriptional activity. To analyze the transcriptional potential of CD2 in more detail, we generated deletion and substitution mutants of this region. Surprisingly, deletion of a 21 aa region of CD2 completely abolished transcriptional activity, and multiple acidic amino acid residues in this region appeared to play a key role in the transcriptional potential of CD2 (Figs. 4–6).

Transcription factors with more than one activation domain can potentially interact with different target proteins in the basal transcription complex [Seipel et al., 1992]. Furthermore, these target proteins may have differential access to activation domains for proximal or distal activation. Distinct activation domains may also regulate different activation pathways, which would result in a synergistic effect on transcriptional activation [Tanaka et al., 1994]. In addition, Q-enriched and acidic activation domains appear to have flexible, modular structures, since tandem activation domains are sufficient to significantly induce transcriptional activity [Seipel et al., 1992; Escher et al., 2000].

In summary, we have identified two distinct transactivation domains in the CD of hNANOG, which function in a synergistic manner to activate transcription in pluripotent hEC NCCIT cells. Furthermore, we identified two well-known classical motifs within these transactivation domains that are required for maximum transcriptional activity of hNANOG in hEC cells. Further investigation is needed to elucidate the mechanism of transcriptional

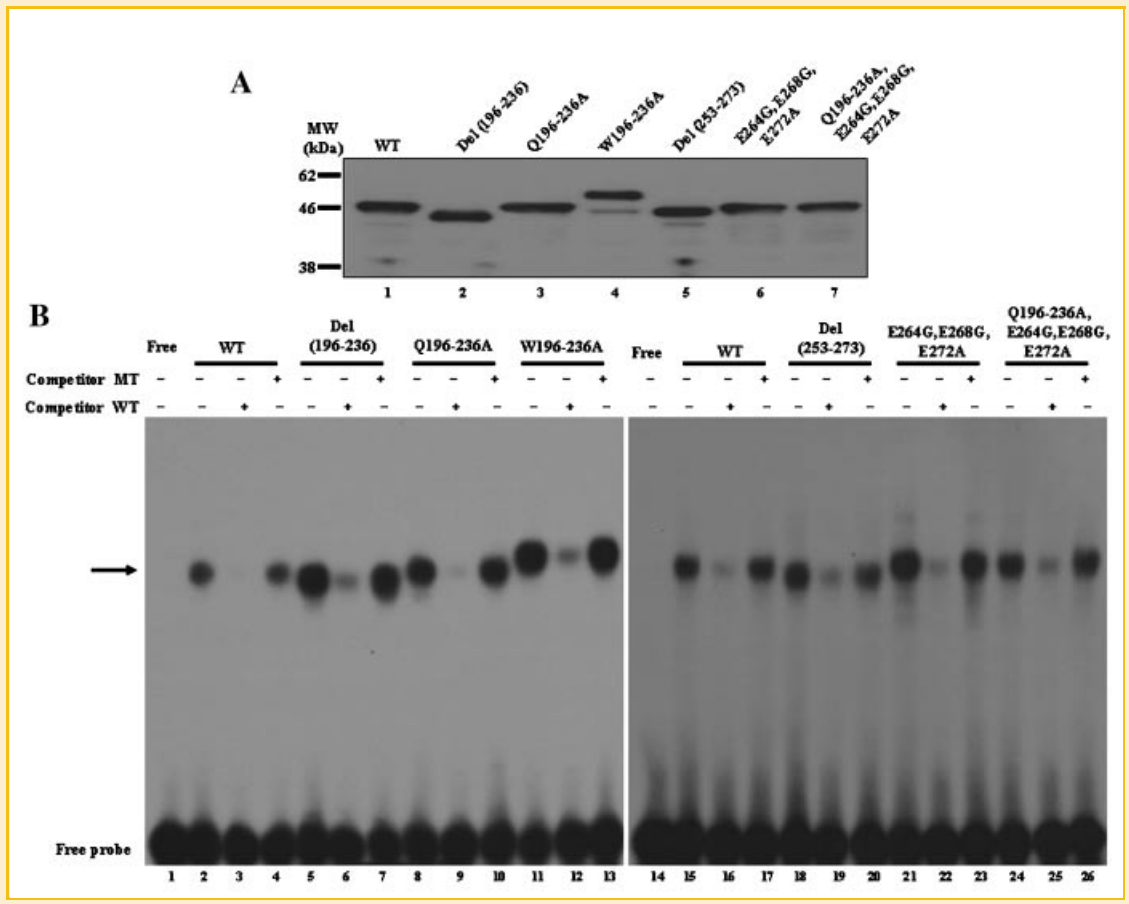

Fig. 8. Reduced transcriptional activity of hNANOG mutants is not due to impaired binding activity. A: Western blot analysis of in vitro transcribed and translated hNANOG WT and mutant derivatives using an anti-Flag antibody. Lanes are as follows: WT (lane 1); Del (196–236) (lane 2); Q196–236A (lane 3); W196–236A (lane 4); Del (253–273) (lane 5); E264G, E268G, E272A (lane 6); Q196–236A, E264G, E268G, E272A (lane 7). B: DNA binding activity of hNANOG WT and mutant derivatives using an oligonucleotide derived from the Nanog binding motif. In vitro transcribed and translated hNANOG WT and mutant proteins were incubated with WT binding site oligonucleotide, either alone or in the presence of 50-fold molar excess of unlabeled WT or mutant (MT) probe as a competitor. Arrow indicates complexes of hNANOG WT or mutant protein and radiolabeled Nanog binding site oligonucleotide. Lanes are as follows: free probe (lane 1 and 14); WT (lanes 2–4 and 15–17); Del (196–236) (lanes 5–7); Q196–236A (lanes 8–10); W196–236A (lanes 11–13); Del (253–273) (lanes 18–20); E264G, E268G, E272A (lanes 21–23); Q196–236A, E264G, E268G, E272A (lanes 24–26).

regulation of downstream target genes by the multiple transactivation domains of hNANOG in GCTs as well as pluripotent germ cells.

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