No Relevance of NF-κB in the Transcriptional Regulation of Human *Nanog* Gene in Embryonic Carcinoma Cells

Hyun Jeong Seok, Young Eun Kim, Jeong-A Park and Younghee Lee†

Dept. of Biochemistry, College of Natural Sciences,
Chungbuk National University, Cheongju 361-763, Korea

ABSTRACT : Embryonic stem (ES) cells can self-renew maintaining the undifferentiated state. Self renewal requires many factors such as Oct4, Sox2, FoxD3, and *Nanog*. NF-κB is a transcription factor involved in many biological activities. Expression and activity of NF-κB increase upon differentiation of ES cells. Reportedly, *Nanog* protein directly binds to NF-κB protein and inhibits its activity in ES cells. Here, we found a potential binding site of NF-κB in the human *Nanog* promoter and postulated that NF-κB protein may regulate expression of the *Nanog* gene. We used human embryonic carcinoma (EC) cells as a model system of ES cells and confirmed decrease of *Nanog* and increase of NF-κB upon differentiation induced by retinoic acid. Although deletion analysis on the DNA fragment including NF-κB binding site suggested involvement of NF-κB in the negative regulation of the promoter, site-directed mutation of NF-κB binding site had no effect on the *Nanog* promoter activity. Furthermore, no direct association of NF-κB with the *Nanog* promoter was detected during differentiation. Therefore, we conclude that NF-κB protein may not be involved in transcriptional regulation of *Nanog* gene expression in EC cells and possibly in ES cells.

Key words : Human *Nanog* gene, Promoter, Negative regulation, NF-κB.

INTRODUCTION

Self-renewal and pluripotency of ES cells are maintained by extrinsic signaling factors and intrinsic factors. Extrinsic signaling factors includes leukemia inhibitory factor (LIF), FGF family members, and TGFβ that are important during feeder-free culture (Daheron et al., 2004; Vallier et al., 2005; James et al., 2005). Intrinsic factors are transcription factors such as Oct4, Sox2, Nanog, Klf4 and others (Pan & Thomson, 2007; Niwa et al., 2000; Silva et al., 2006; Babaie et al., 2007; Jaenisch & Young, 2008; Chan et al., 2009). Nanog protein is expressed in inner cell mass of blastocyst and is considered as an important transcription factor for self-renewal and pluripotency in ES cells. The human and mouse Nanog proteins have similarity of 69%. Overexpression of Nanog can maintain self-renewal capacity and pluripotency of mouse ES cells in the absence of LIF. When a null mutation of Nanog was introduced, ES cells differentiate mainly to visceral and parietal endoderm cells (Chambers et al., 2003; Mitsui et al., 2003). Therefore, Nanog seems to be a necessary and sufficient factor for the maintenance of ES cells. In contrast, low expression or overexpression of Oct4 leads to differentiation of ES cells suggesting that fine regulation of Oct4 expression level is critical for self renewal of ES cells. Nanog protein activates genes involved in the maintenance of stem cell properties and suppresses the genes involved in differentiation or terminally differentiated cell properties.

Recent study on the murine *Nanog* promoter revealed two transcription initiation start sites and the importance of Oct1 binding for promoter activity (Wu & Yao, 2005). The *Nanog* promoter is known to be activated by Oct4 and Sox2, whereas it is suppressed by GATA6 and p53 (Niwa

† Corresponding author: Younghee Lee, Dept. of Biochemistry, Chungbuk National University, 410 Sungbong-ro, Heungduk-gu, Cheongju 361-763, Korea. Tel: +82-43-261-3387; Fax: +82-43-267-2306, E-mail: yhl4177@cbnu.ac.kr

et al., 1998; Loh et al., 2006; Seok et al., 2010). These results suggest a complexity in the control of *Nanog* gene expression.

NF-κB is a transcription factor involved in many biological activities. Previously, we reported that expression and activity of NF-κB in human and mouse ES cells is lower than that of differentiated cells, and suggest that NF-κB may be involved in differentiation rather than self-renewal (Kang et al., 2007; Kim et al., 2008). Other investigators also reported that Nanog protein maintains pluripotency of ES cells by direct association and suppression of NF-κB (Torres & Watt, 2008). They also showed that enforced expression of NF-κB promotes differentiation of ES cells (Torres & Watt, 2008).

Here, we found a putative binding sites of NF-κB and c-Rel in the *Nanog* promoter and examined whether NF-κB may control expression of Nanog gene in human EC cell line NCCIT. Our results suggested that NF-κB is not involved in the transcriptional regulation of *Nanog* gene.

MATERIALS AND METHODS

1. Culture of Cells and Transfection

Human EC cell line NCCIT was obtained from American Type Culture Collection (ATCC, Manassas, USA). Cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, USA), supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), non-essential amino acid (Invitrogen, USA) and penicillin, at 37℃ in 5% CO_2 (5×10^5 cells/10 cm dish). Cells were seeded at 5×10^5 cells per well in 6 well plates 24 h before transfection. Each promoter reporter constructs (4 ㎍) were co-transfected with pRL-null vector as an internal control using Lipofectamine 2000 (Invitrogen). If necessary, NCCIT cells were treated with 10^{-5} M retinoic acid to induce differentiation (all-*trans*, Sigma-Aldrich, USA).

2. Construction of Reporter Vectors

Serially deleted *Nanog* promoter constructs were prepared as described previously (Seok et al., 2010). In brief, partial fragments of the human *Nanog* promoter were PCR amplified using the human genomic DNA as a template. The promoter sequence was subcloned into pGL3-basic vector (Promega, USA). Point mutation was introduced in the binding site of NF-κB by site-directed mutagenesis. We performed two-step PCR with the primers for mutation and the PCR products were cloned using T-Blunt cloning kit (Solgent, Korea). The mutated promoter sequence was cloned into pGL3-basic vector using Kpn Ⅰ and Xho Ⅰ sites. The primers used for mutation are 5'-CAGGTTCAAGCGATT CTCCCGCCTCA-3' and 5'-CGGGAGAATCGCTTGAAC CTGGGAGG-3'.

3. Luciferase Reporter Assay

Cells transfected with different reporter constructs were lysed with 500 ㎕ of lysis buffer (Promega) 24 h after transfection. 20 ㎕ aliquots of the cell lysates were subjected to luciferase activity assay using Dual Luciferase Reporter (DLR) assay system (Promega) according to the supplier's recommendation. We used cells transfected with pGL3-basic vector as a negative control. Luciferase activity was measured by Junior LB 9509 (Berthold, Germany).

4. Western Blotting

Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (Millipore Corp, USA). To block nonspecific binding, membranes were treated with 5% skim milk in Tris buffered saline-Tween 20 (TBS-T; 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) for 1 h. The membranes were probed with anti-Oct4 (Santa Cruz Biotechnology, USA), anti-Nanog (Millipore Corp), anti-GAPDH (Santa Cruz Biotechnology), or anti-NF-κB (Delta Biolabs, UK) antibodies. HRP-conjugated anti-rabbit (Amersham Pharmacia Biotech, USA) or anti-mouse secondary antibodies (Santa Cruz Biotechnology) were applied and the immuno-reactivity was detected with West-Zol™ Plus (iNtRON Biotechnology, Korea).

5. Chromatin Immunoprecipitation (ChIP)

Cells were cultured to grow approximately 80% confluence in 10 cm dish. 5×10^6 EC cells or differentiated cells were fixed with 1% formaldehyde at room temperature for 10 min and neutralized by the addition of 125 mM glycine for 5 min. Cells were washed twice in ice- cold PBS and lysed in 1 ㎖ of lysis buffer (Tris-EDTA (TE) buffer (pH 8.0), 0.5% NP-40, protease inhibitors). After centrifugation, the pellets were washed twice in ice-cold PBS, resuspended in nuclei lysis buffer (TE, 1% SDS, protease inhibitors), and sonicated to an average fragment size of 0.6 kb. The solution was centrifuged at 14,000 rpm for 10 min. Supernatant was precleared with normal mouse IgG and protein A-agarose beads (Santa Cruz Biotechnology). Soluble chromatin sample was immunoprecipitated with anti-NF-κB (Delta Biolabs) or anti-Oct4 (Santa Cruz Biotechnology) or an equal amount of rabbit or mouse IgG (Santa Cruz Biotechnology). After DNA and protein complexes were dissociated by reverse cross-linking in 12 ㎖ of 5M NaCl at 65℃ for 4-5 h, proteins were removed by extraction with phenol/chloroform/isoamyl alcohol. Immunoprecipitated DNA samples were subjected to PCR using the *Nanog* promoter specific primers 5'-AGGCGGATCA C CTGAAGTC-3' and 5'-ACCTTAGACCCACCCCTCCT-3'. The DNA purified from the sonicated nuclear lysates was directly analyzed by PCR using the same primer set, which was used as an input control.

RESULTS AND DISCUSSION

1. Expression of NF-κB and *Nanog* during Differentiation of Embryonic Carcinoma Cells

Previously, we and others reported that NF-κB associates with differentiation and that Nanog protein maintains pluripotency of ES cells by inhibiting NF-κB (Kang et al., 2007; Kim et al., 2008; Torres & Watt, 2008). Here, we postulated that NF-κB may control expression or activity of Nanog in reverse. As the efficiency of transfection is low in ES cells, we used human EC cells NCCIT in this study. We first confirmed that NF-κB increased upon differentiation when we induced differentiation of EC cells using retinoic acid (Fig. 1). Oct4 and Nanog, which are pluripotency markers of ES cells and EC cells, decreased during differentiation of EC cells. While Nanog protein was undetectable three days after treatment with retinoic acid, Oct4 gradually reduced up to 5 days of differentiation. As expression and regulation of the human *Nanog* gene seem to be similar in ES cells and EC cells, we further studied regulation of the human *Nanog* gene expression in EC cells.

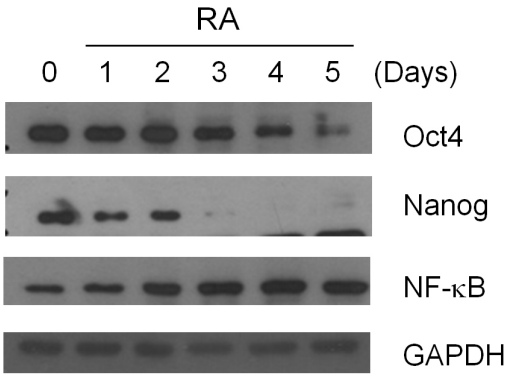

Fig. 1. Increase of NF-κB protein in NCCIT cells during differentiation. Differentiation of NCCIT cells was induced by treatment with 10^{-5} M retinoic acid (RA) for 5 days. Expression levels of NF-κB, Oct4 and *Nanog* proteins were examined by Western blotting analysis. GAPDH served as a loading control.

2. Production and Analysis of Various Deletion or Mutant Promoter Constructs

We analyzed the putative transcription factor binding sites within the 1.6 kb promoter region of human *Nanog* promoter using MatInspector program (http://www.genomatix.de) and TFSEARCH (ver 1.3) (http://www.cbrc.jp/research/db/TFSEARCH.html). We found that the 1.0 kb *Nanog* promoter region includes a NF-κB p65 binding site (−488/−500) and a c-Rel binding site (−1163/−1173). Previously, we cloned serially deleted sequences of *Nanog* promoter by PCR amplification using genomic DNA as a template

and constructed various *Nanog* promoter constructs for activity assay using pGL3 as a parental vector (Seok et al., 2010). We used the constructs to determine the contribution of NF-κB family members to the transcriptional regulation of human *Nanog* promoter. After transfection of the promoter constructs (0.8 kb, 1 kb, 1.2 kb, 1.4 kb) to NCCIT cells, we performed luciferase activity assays and compared the promoter activity of the constructs. There was an increase of promoter activity in 0.8 kb compared with the others, which implies that the 0.2 kb DNA fragment that is missing in 0.8 kb construct has a negative regulatory element (Fig. 2). As the putative NF-κB p65 binding site (−488/−500) resides in the 0.2 kb DNA fragment, NF-κB p65 can be a candidate negative regulator.

To identify whether NF-κB and NF-κB p65 binding site are involved in the negative regulation, a site-directed mutation was introduced in the putative NF-κB p65 binding site using the 1.0 kb human *Nanog* promoter construct as a template (Fig. 3A). The point mutation was designed on the sequence which is usually used as a mutant oligonucleotide for NF-κB binding in gel-shift assay. As shown in Fig. 3B, mutation of the NF-κB p65 binding site doesn't affect the activity of the 1.0 kb *Nanog* promoter. Therefore, it is likely that NF-κB binding site is not involved in regulation of *Nanog* gene expression in EC cells and possibly in ES cells. As there were several putative transcription factor binding sites in the 0.2 kb DNA fragment that is missing in 0.8 kb construct, some of them may contribute to negative regulation of the *Nanog* promoter in ES cells.

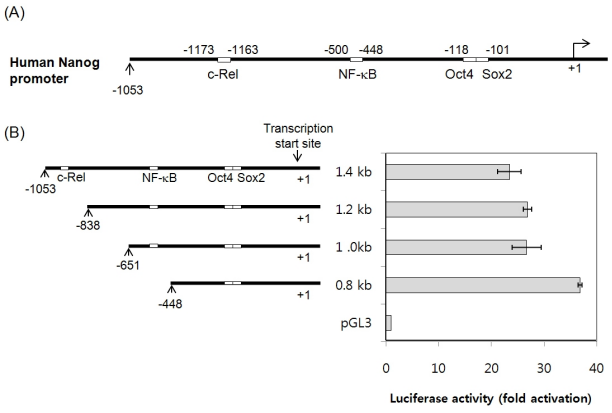

Fig. 2. Analysis of serially deleted mutant constructs of the human *Nanog* promoter. A) Schematic representation of the *Nanog* promoter and predicted NF-κB family binding sites. Putative binding sites for NF-κB p65, c-Rel, and Oct4 and Sox2 are indicated. B) Luciferase activity of the serially deleted mutant promoter constructs. Promoter-reporter constructs were transfected to NCCIT cells along with pRL-null vector. After 24 hr incubation, luciferase activity was measured and normalized with pRL-null activity. Luciferase activity is shown as a fold-activation compared with the cells transfected with pGL3-basic as a vector control. This result represent mean fold activation from three independent experiments.

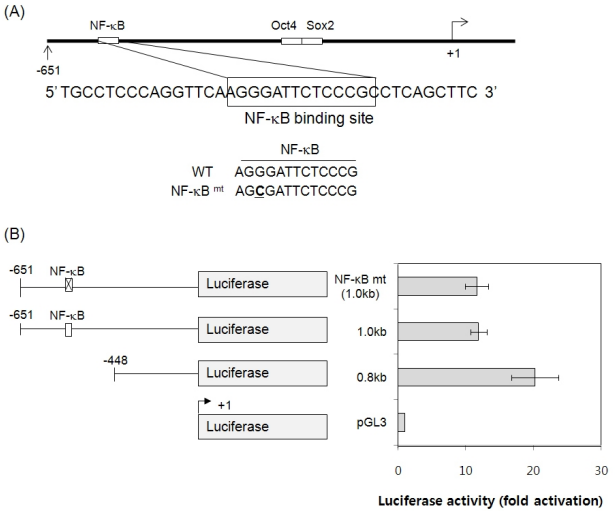

Fig. 3. Construction and analysis of the mutant *Nanog* promoter construct which has a site-directed mutation in the NF-κB p65 binding site. A) Site-directed mutagenesis. Mutation was introduced into the 1.0kb human *Nanog* promoter. Change of the nucleotide is indicated in bold underlined letter. B) Effect of NF-κB p65 binding site mutation on the *Nanog* promoter activity. NCCIT cells were transfected with wild type (1.0kb) or mutant promoter (NF-κBmt). Cell lysates were prepared and the luciferase activity was measured and normalized with pRL-null activity. Luciferase activity is shown as a fold-activation compared with the cells transfected with pGL3-basic as a vector control. This result represent mean fold activation from four independent experiments.

3. No Association of NF-κB with the Human *Nanog* Gene Promoter during Differentiation of EC Cells

Previously, it was shown that Oct4 and Sox2 are necessary for major *Nanog* promoter activity in human and mouse ES cells (Wu & Yao, 2005; Babaie et al., 2007; Rodda et al., 2005). Considering that NF-κB expression is comparatively low in ES cells and that Nanog inhibits the function of NF-κB in ES cells (Kang et al., 2007; Kim et al., 2008; Torres & Watt, 2008), it is not surprising that NF-κB does not bind to the *Nanog* promoter in ES cells and EC cells. Therefore, we decided to investigate whether NF-κB is involved in the rapid decrease of Nanog expression during differentiation of EC cells. We treated NCCIT cells with retinoic acid and performed ChIP assay using anti-NF-κB antibody to examine association of NF-κB with the human *Nanog* promoter. As shown in Fig. 4, no binding of NF-κB was found in undifferentiated NCCIT cells (0 day) or in the cells during differentiation. In contrast, binding of Oct4 to the *Nanog* promoter was confirmed in undifferentiated NCCIT cells (data not shown). Taken together with the results from deletion or mutant promoter constructs (Fig. 2 and 3), this result confirms that NF-κB is not involved in the transcriptional regulation of the human *Nanog* promoter even during differentiation induced by retinoic acid.

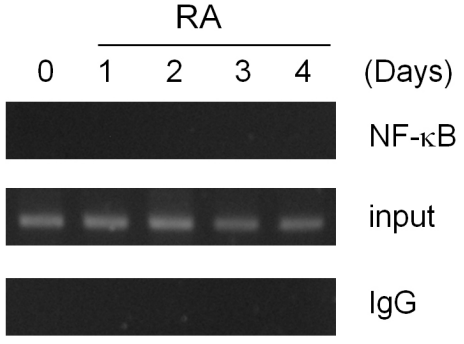

Fig. 4. NF-κB does not bind to the human *Nanog* promoter. Soluble chromatin from NCCIT cells was coimmunoprecipitated with anti-NF-κB antibody, anti-Oct4 antibody or equal amount of rabbit or mouse IgG. The immunoprecipitates were subjected to PCR analysis using *Nanog* promoter specific primers. The DNA purified from the sonicated nuclear lysates was analysed as an input control.

NF-κB is involved in many biological activities including inflammation, survival, and differentiation (Denk et al., 2000; Qiu et al., 1998). In ES cells, NF-κB is considered as a factor associated with differentiation (Kang et al., 2007; Kim et al., 2008; Torres & Watt, 2008). Even though Nanog protein is known to suppress the activity of NF-κB in ES cells, the exact role of NF-κB in differentiation is not yet known. As Nanog protein decreases when ES or EC cells differentiate, we checked the possibility that NF-κB directly controls the expression of the human *Nanog* gene at the transcriptional level in human EC cells. However, our overall results suggest that NF-κB is not binding to the *Nanog* promoter and is not involved in the transcriptional regulation of the *Nanog* promoter. As Nanog protein is known to directly associate with NF-κB, we can not exclude the possibility that the protein-protein interaction contributes to decrease of Nanog during differentiation. In conclusion, the mechanism by which NF-κB contributes to differentiation of ES cells is still unclear and to be clarified in the future.

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