Differential transcriptional regulation of the \textit{NANOG} gene in chicken primordial germ cells and embryonic stem cells

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Abstract

\textbf{Background:} \textit{NANOG} is a core transcription factor (TF) in embryonic stem cells (ESCs) and primordial germ cells (PGCs). Regulation of the \textit{NANOG} gene by TFs, epigenetic factors, and autoregulatory factors is well characterized in ESCs, and transcriptional regulation of \textit{NANOG} is well established in these cells. Although \textit{NANOG} plays a key role in germ cells, the molecular mechanism underlying its transcriptional regulation in PGCs has not been studied. Therefore, we investigated the mechanism that regulates transcription of the chicken \textit{NANOG} (\textit{cNANOG}) gene in PGCs and ESCs.

\textbf{Results:} We first identified the transcription start site of \textit{cNANOG} by 5' rapid amplification of cDNA ends PCR analysis. Then, we measured the promoter activity of various 5' flanking regions of \textit{cNANOG} in chicken PGCs and ESCs using the luciferase reporter assay. \textit{cNANOG} expression required transcriptional regulatory elements, which were positively regulated by \textit{POU5F3 (OCT4)} and \textit{SOX2} and negatively regulated by \textit{TP53} in PGCs. The proximal region of the \textit{cNANOG} promoter contains a positive transcriptional regulatory element (CCAAT/enhancer-binding protein (CEBP)-binding site) in ESCs. Furthermore, small interfering RNA-mediated knockdown demonstrated that \textit{POU5F3}, \textit{SOX2}, and \textit{CEBP} played a role in cell type-specific transcription of \textit{cNANOG}.

\textbf{Conclusions:} We show for the first time that different trans-regulatory elements control transcription of \textit{cNANOG} in a cell type-specific manner. This finding might help to elucidate the mechanism that regulates \textit{cNANOG} expression in PGCs and ESCs.

\textbf{Keywords:} Chicken, Embryonic stem cells, \textit{NANOG} gene, Primordial germ cells, Regulatory elements

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Background

Gene transcription is mainly regulated by transcription factors (TFs) that bind to specific DNA sequences (called motifs) located in the promoter regions of genes [1]. Many TFs contribute to tissue- and cell type-specific gene transcription according to their recognition specificity [2–4]. In addition, TFs generally initiate and guide cell fate such as lineage progression and control the stability of cell differentiation [5]. Therefore, identification of regulatory elements within the promoter region is considered crucial to understand the mechanism underlying transcriptional regulation in specific cell types. A germ cell-specific gene regulatory network is required to maintain the unique properties of primordial germ cells (PGCs) for transmission of genetic information to the next generation [6]. Many studies have investigated germ cell-specific gene promoters to understand their regulatory mechanisms. In many species, germ cells have a unique mechanism of transcription initiation that uses alternate forms of core promoter elements [7–10]. Also, germ cells reorganize different type of core promoter TFs under the control of germ cell-specific TFs during germ cell differentiation [11–13].

In mammals, core TFs such as NANOG, OCT4, and SOX2 control maintenance of pluripotency. Core TFs play an important role in establishing control of gene expression programs that define the identity of embryonic stem cells (ESCs) [14–16]. In particular, the NANOG gene is important for acquisition of pluripotency by ESCs and embryonic germ cells (EGCs) [17–19]. Several earlier studies identified the regulatory elements of NANOG that are required to maintain the self-renewal and pluripotency of ESCs [20–22]. The major regulators of NANOG expression are Octamer- and Sox-binding elements present at the upstream of transcription start site (TSS) in its promoter region, and these elements are positively regulated by binding of OCT4 and SOX2 in ESCs [20, 23]. Direct binding of ZFP143 to the proximal region of the NANOG promoter regulates NANOG expression by modulating OCT4 binding [24]. In addition, TF-binding cis-regulatory elements of NANOG, including SP1/SP3−, SALL4−, and BRD4-binding sites, have been identified as positive regulators [25–27]. On the other hand, P53-binding sites negatively regulate NANOG expression to induce differentiation of ESCs [28]. Therefore, regulation of NANOG expression plays a critical role in determining the fate of pluripotent cells.

PGCs express several pluripotency-related TFs such as NANOG, POLISF3, and SOX2, and their expression controls transcription of germness-related genes in these cells [11, 29]. NANOG plays an essential role during early germ cell development as a key TF required for the formation of PGCs and maintenance of early germ cells [30, 31]. NANOG-deficient PGCs reportedly undergo apoptotic death [32]. It was recently reported that NANOG regulates PGC-specific epigenetic programming and global histone methylation [33, 34]. NANOG is evolutionarily conserved in mammals and most of the lower vertebrate species, including chicken. In particular, NANOG orthologs from chicken, zebrafish, and axolotl are highly conserved [35–37]. Similar to mammals, NANOG is crucial to maintain pluripotency and self-renewal of chicken ESCs [35]. NANOG is expressed during chicken intrauterine embryonic development and is exclusively expressed in PGCs from Hamburger and Hamilton stage 5 (HH5) to HH8. Therefore, NANOG is also important to maintain pluripotency and cell proliferation in chicken intrauterine embryos and PGCs [31, 35, 38]. Despite the exclusive expression of NANOG in chicken PGCs, the molecular mechanism that regulates its transcription in these cells has not been fully clarified. This study investigated enhancers and suppressors of the proximal promoter region of the chicken NANOG (cNANOG) gene in PGCs and ESCs. Furthermore, we investigated transcriptional control of cNANOG expression via trans-regulatory elements and TFs, which are important for its cell type-specific expression.

Methods

Experimental design, animals, and animal care

This study investigated the cis- and trans-regulatory elements that are important for modulating transcription of the NANOG gene in chicken PGCs using the dual luciferase assay and transcriptome analysis. The management of White Leghorn (WL) chickens was approved by the Institute of Laboratory Animal Resources, Seoul National University, Korea (SNU-190401-1-1). The chickens were housed according to standard procedures at the University Animal Farm, Seoul National University, Korea.

5' Rapid amplification of cDNA ends (5'-RACE) PCR analysis

To determine the TSS of the cNANOG gene (Gene ID: 100272166), 5'-RACE PCR was performed using a GeneRacer Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Gene Racer RNA Oligo-ligated mRNA was reverse-transcribed into cDNA. Single-stranded cDNA served as the template in nested 5'-RACE PCR using the GeneRacer 5' Primer and reverse gene-specific primers (GSPs). The cNANOG reverse GSP was 5'-GTC TGC AGT AGG GCT AGT GGC AGA GTC T-3'. The RACE products were identified by DNA sequencing analysis. To confirm the quality of adapter-ligated RNA, 5'-RACE PCR was performed with a chicken β-actin reverse GSP, which was 872 bp in size and contained 828 bp of β-actin and 44 bp of the GeneRacer RNA Oligo.
reporter assays were repeated at least three times. An empty vector, was used as a negative control. All determining the ratio of Nluc/Fluc signals in AU. pNL1.2, System; Promega). Promoter activities were calculated by measured using a luminometer (Glomax-Multi-Detection Signals in arbitrary units (AU) of Nluc and Fluc were quenched, followed by reaction with Nluc substrate. Fluc signals were then transfected for 24 h, cells were lysed with lysis buffer containing Fluc substrate. Fluc signals were then measured using a luminometer (Glomax-Multi-Detection System; Promega). Promoter activities were calculated by determining the ratio of Nluc/Fluc signals in AU. pNL1.2, an empty vector, was used as a negative control. All reporter assays were repeated at least three times.

Table 1 List of primer sequences used to clone the NANOG promoter

| Primer name        | Primer sequence (5′ → 3′)          |
|--------------------|-----------------------------------|
| cNANOG – 3550 bp_F | AAGCTTTGCTTCCTTCTGACC             |
| cNANOG – 3375 bp_F | CTGGAGCTAAGGCGGTCG               |
| cNANOG – 3154 bp_F | TGGGCCCTCTGTTACAGCT              |
| cNANOG – 2928 bp_F | CGACGAGTCAAGCAGCTCCGAA           |
| cNANOG – 1988 bp_F | GCGACACGTGGAAACA                 |
| cNANOG – 945 bp_F  | CATGGGGTGTTGTGCTCTGCT            |
| cNANOG – 627 bp_F  | CTCTTTTGTCCTCCTCC                |
| cNANOG – 442 bp_F  | CTGCAGTCTGCTCAATGCG              |
| cNANOG – 407 bp_F  | AAGTCCCGGGGCGTTCTCTGG            |
| cNANOG – 377 bp_F  | CCATTCTTGTACTGGTGAGGACCGATGAG    |
| cNANOG – 312 bp_F  | CGAGGGGCGGTTGCTCCCGAGCGCCAG      |
| cNANOG – 250 bp_F  | CTGCAGTCTGCTCCTCC                |
| cNANOG – 210 bp_F  | CTGCAGTCTGCAATGCG                |
| cNANOG – 170 bp_F  | CCAAGGGGAGAAAGCGTG               |
| cNANOG – 130 bp_F  | AACTTCCGGATATCCCTATCATGCAGC      |
| cNANOG – 69 bp_F   | TCCTGACACTCCCTGTC                |
| cNANOG promoter_R  | GGTCGGGAGCAGACCT                 |

Construction of NanoLuc luciferase expression vectors derived from the cNANOG promoter

To construct NanoLuc luciferase expression vectors, the 5′ flanking region of the cNANOG gene was amplified using genomic DNA extracted from adult chicken blood and inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA). Primer sets were used to clone differently sized fragments of the cNANOG promoter (Table 1). Then, different lengths of the 5′ upstream region of the cNANOG gene were inserted between the KpnI and XhoI sites of the pNL1.2 vector (Promega).

Luciferase reporter assay

The Nano-Glo Dual Reporter Assay System (Promega) was used to assess cNANOG promoter activity. Prepared cells were seeded in a 96-well plate and co-transfected with the pGL4.53 firefly luciferase (Fluc) and pNL1.2 (NlucP/cNANOG RE) NanoLuc luciferase (Nluc) plasmids using Lipofectamine 2000 (Invitrogen). After transfection for 24 h, cells were lysed with lysis buffer containing Fluc substrate. Fluc signals were then quenched, followed by reaction with Nluc substrate. Signals in arbitrary units (AU) of Nluc and Fluc were measured using a luminometer (Glomax-Multi-Detection System; Promega). Promoter activities were calculated by determining the ratio of Nluc/Fluc signals in AU. pNL1.2, an empty vector, was used as a negative control. All reporter assays were repeated at least three times.

Table 2 List of siRNA sequences targeting each transcription factor for knockdown analysis

| Target gene | siRNA sequence (5′ → 3′) | Sense | Antisense |
|-------------|---------------------------|-------|-----------|
| POU5F3      | UGGCUCUAUAGGCGAGGAGGA     | UCCUCGCCCAGAUGGCGCA |       |
| SOX2        | AAAACAGCCCGAGGAGGAGGA     | AAGGUGGAGGAGGAGGAGGA |       |
| TP53        | CAAUGGACUCUCGAGCAGCU      | UUGGCUAGGAGGAGGAGGAGG |       |
| CEBPA       | GCGAGGAGGAGGAGGAGGAGG     | UUGGCUAGGAGGAGGAGGAGG |       |
| CEBPB       | GCGAGGAGGAGGAGGAGGAGG     | UUGGCUAGGAGGAGGAGGAGG |       |
| CEBPD       | ACGAGAAGCUCGAGCAAGAGA     | UUGGCUAGGAGGAGGAGGAGG |       |
| CEBPG       | AAAUAAGCUCUCGAGCAAGCA     | UUGGCUAGGAGGAGGAGGAGG |       |
| CEBPZ       | GAGAAAGCAGAGGAGGAGG       | UUGGCUAGGAGGAGGAGGAGG |       |
Prediction of putative TF-binding elements

TF-binding sites were predicted by MatInspector, a Genomatix program (http://www.genomatix.de/) using TRANSFAC matrices (vertebrate matrix; core similarity 1.0 and matrix similarity 0.8), and PROMO 3.0, which uses TRANSFAC version 8.3 (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

Small interfering RNA (siRNA)-mediated knockdown of predicted TFs

siRNAs targeting predicted TFs were designed using siRNA Target Finder (http://www.ambion.com) (Table 2). Commercially available control siRNA (sense: 5′-CCU AGC CCA AUU UCG U-3′) was purchased from Bioneer Corporation (Daejeon, Korea). To validate the knockdown efficiency of predicted TFs, PGCs or ESCs were transfected with 50 pmol of siRNAs targeting CCAAT/enhancer-binding protein (CEBP) genes, including CEBPA, CEBPB, CEBPD, CEBPG, and CEBPZ, and TP53 using Lipofectamine 2000 (Invitrogen). After siRNA transfection for 24 h, the knockdown efficiencies of the predicted TFs and the effects on cNANOG gene transcription were measured by quantitative reverse-transcription PCR (RT-qPCR).

Analysis of gene expression by RT-qPCR

Total RNA was extracted from test samples using TRIzol reagent (Molecular Research Center, USA) in accordance with the manufacturer’s protocol and reverse-transcribed using the Superscript III First-Strand Synthesis System (Invitrogen). The PCR mixture contained 2 μL of PCR buffer, 1 μL of 20× EvaGreen qPCR dye (Biotium, Hayward, CA, USA), 0.4 μL of 10 mmol/L dNTP mixture, and 10 pmol each of gene-specific forward and reverse primers (Table 3). RT-qPCR was performed in triplicate. Relative

Table 3 List of primer sequences used for quantitative real-time PCR

| Gene symbol | Primer sequence (5′ → 3′) | Forward | Reverse |
|-------------|---------------------------|---------|---------|
| CEBPA       | CCCACCTGCACTACACAGATC     | TCTTTTGGATTTGCGGCGG |         |
| CEBPB       | GCCGCGCGCTTATACCTCATG     | GGCTGAAGTCAAGCTGCCT |         |
| CEBPD       | ACTCTAGCCACGCAAGGTTG     | CTGCTCCTGTATACATGAC |         |
| CEBPG       | CCCGACGAGTCGTCGAGTGT     | GGACGGGCTCTCTCTGAC  |         |
| CEBPZ       | GGCTGCTACAGTGCTCCACT     | GGACGCCGTGAGAGAACCA |         |
| SOX2        | AAACCGGCACTGAAACCTCC    | TGTGACATCTCCGAGTTTCC |         |
| SOX3        | CGGCTCAGCAGTCTCGACTAC   | TCGGCTGAGGCTTAAAGAC |         |
| POU5F3      | TAGGGGAAACCTCGGAGAGC   | ATGGTCACTGGGATGGGCA |         |
| TP53        | CCGTGCGCGCTCTATAAGAGA | ACAGCACGGTGACAGA   |         |
| NANOG       | AGTGGGTGCTAAGGGTATTAC  | ACTACTGCTGCCCTCCTAG |         |
| GAPDH       | GTGGTGCTAAGCGGTTAT     | ACCTCTGACATCCTCCT GA |         |

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target gene expression was quantified after normalization against chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression as an endogenous control.

Statistical analysis
Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Significant differences between groups were determined by a one-way analysis of variance with Bonferroni’s multiple comparison test and the unpaired t-test. A value of \( P < 0.05 \) indicated statistical significance.

Results
Identification of the TSS of the cNANOG gene
To better understand transcriptional regulation of the cNANOG gene, we first determined the TSS of this gene by 5′-RACE PCR analysis. A 470 bp PCR product was obtained using a reverse GSP that targeted exon 2 of the cNANOG gene (Fig. 1a and b). Sequencing analysis identified the TSS of the cNANOG gene located 70 bp upstream of the ATG start codon (Fig. 1b).

Characterization of the cNANOG core promoter in PGCs and ESCs
To investigate the proximal region of the core promoter of the cNANOG gene, we generated a series of 5′ deletion luciferase reporter constructs of the 6− region, which were randomly designed based on the −3550/+70 bp sequence (Fig. 2a). Luciferase activity derived from differently sized fragments of the cNANOG promoter was examined in PGCs, ESCs, and DF-1 cells transfected with the constructs for 24 h using Lipofectamine 2000. Luciferase activity was 4-fold higher in PGCs transfected with the −3550/+70 bp fragment than in PGCs transfected with the −250/+70 bp fragment (Fig. 2b). On the other hand, the −250/+70 bp fragment did not exhibit luciferase activity in ESCs (Fig. 2c). None of the cNANOG promoter fragments were active in DF-1 cells (Fig. 2d). These results suggest that transactivation level of the complete promoter (−3550/+70 bp sequence) was similar between PGCs and ESCs but cNANOG transcription was differentially regulated in PGCs and ESCs by the proximal enhancer.

POUSF3 and SOX2 regulate constitutive expression of cNANOG in PGCs
To further examine PGC-specific cNANOG promoter activity and binding to the proximal enhancer, we generated four constructs harboring fragments of the −250/+70 bp region of the cNANOG promoter via deletion of the 5′ upstream region. Among the four constructs, the −210/+70 bp, −170/+70 bp, and −130/+70 bp fragments still showed promoter activity in PGCs, while the −69/+70
bp fragment did not (Fig. 3a). None of the cNANOG promoter fragments were active in DF-1 cells (Fig. 3b). These results suggest that a positive transcriptional regulatory element is located between $-130$ and $-69$ bp in PGCs.

Based on the findings regarding cNANOG promoter activity described above, we predicted TFs with binding sites located between $-130$ and $-69$ bp of the cNANOG promoter using two software programs (PROMO and MatInspector). Several TF-binding sites, including AIRE-, NFy-, CMYB-, ISL1-, E2F-, and OSNT (OCT4/POUSF3, SOX2, NANOg, and TCF3)-binding sites, were identified in this region (Fig. 3c). Sequence alignment of this cNANOG promoter region from six vertebrate species showed that the POU5F3- and SOX2-binding regulatory elements are highly conserved in mammalian species (Fig. 3d). To determine the functional contributions of the POU5F3- and SOX2-binding sites to constitutive expression of cNANOG, site-directed mutagenesis, which can disturb the recruitment of TFs, was performed (Fig. 3e). Mutation of the POU5F3/SOX2-binding sites in the 200 bp fragment ($-130/70$ bp) significantly reduced relative luciferase activity in PGCs. Moreover, relative luciferase activity was reduced significantly more by mutation of the SOX2-binding site

**Fig. 3** Verification of the proximal enhancer of the chicken NANOG (cNANOG gene) in chicken primordial germ cells (PGCs). a-b Schematic diagram of the constructed cNANOG promoter vectors and luciferase activity in PGCs (a) and DF-1 cells (b). c Prediction of transcription factor (TF)-binding sites in the cNANOG promoter region located from $-250$ to $+70$ bp. d Multiple alignment of the putative cNANOG proximal enhancer with transcriptional regulatory elements of NANOg genes from mouse, rat, human, cattle, sheep, pig, and chicken. Prediction of mostly conserved POU5F3- and SOX2-binding sites in chicken. e Mutation analysis of putative POU5F3- and SOX2-binding sites in PGCs. f Luciferase activity of the $-130/70$ bp cNANOG promoter fragment compared with that of mutated promoter constructs. Significant differences are indicated as **P < 0.01 and ***P < 0.001. Error bar represent the SEs for five replicate reactions.
alone than by mutation of the *POUSF3*-binding site alone in PGCs (Fig. 3f). Taken together, these results suggest that *POUSF3* and *SOX2* play a role in transcription of *cNANOG* by directly binding to the 5′ upstream promoter region in PGCs.

**TP53 suppresses cNANOG gene expression in PGCs**

Luciferase activity was at least 3-fold higher in PGCs transfected with the −210/+70 bp, −170/+70 bp, and −130/+70 bp fragments than in PGCs transfected with the −250/+70 bp fragment (Fig. 3a). These results suggest that a negative transcriptional regulatory element is located between −250 and −210 bp. To investigate the suppression of *cNANOG* promoter activity, we predicted TFs that have binding sites within this region using two software programs (PROMO and MatInspector) (Fig. 4a). Among the predicted TFs, *TP53* is a suppressor of *NANOG* transcription, while *ZIC2/3* and *CEBP* are

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**Fig. 4** Negative regulation of chicken *NANOG* (*cNANOG*) gene expression by *TP53* in chicken primordial germ cells (PGCs). **a** Prediction of transcription factor (TF)-binding sites in the *cNANOG* promoter region from −250 to −210 bp. **b** Mutation analysis of putative *TP53*-binding sites in PGCs. **c** Luciferase activity of pNL-*NANOG* − 250/+70 and *TP53*-binding site-mutated (pNL-*NANOG* − 250/+70 *TP53* mutation) vectors. pNL1.2-Basic was used as a control. Significant differences are indicated as ns (no significance) and *** *P* < 0.001. Error bar represent the SEs for five replicate reactions.
positive regulators of NANO\textsubscript{G} transcription [28, 40, 41]. We further examined whether TP53 affects cNANO\textsubscript{G} promoter activity in PGCs by performing site-directed mutagenesis and comparing the mutant with the wild-type −250/+70 bp fragment (Fig. 4b). Deletion of the TP53-binding site in the cNANO\textsubscript{G} promoter region significantly increased luciferase activity in PGCs (Fig. 4c). These results demonstrate that TP53 suppresses cNANO\textsubscript{G} transcription in PGCs.

\textbf{CEBP transactivates the cNANO\textsubscript{G} promoter in ESCs}

To further investigate the potential transcriptional regulatory elements in ESCs, we generated four constructs harboring fragments of the −442/+70 bp region of the cNANO\textsubscript{G} promoter via deletion of the 5′ upstream region. Among the four constructs, the −407/+70 bp, −377/+70 bp, and −312/+70 bp fragments exhibited significantly reduced cNANO\textsubscript{G} promoter activity in PGCs (Fig. 4c). These results demonstrate that TP53 suppresses cNANO\textsubscript{G} transcription in PGCs.

We analyzed the −442/+70 bp fragment using two software programs (PROMO and MatInspector) to identify important TF-binding sites that maintain the basal activity of the cNANO\textsubscript{G} gene in ESCs. Only a CEBP-binding site was identified between −462 and −407 bp (Fig. 5c). To examine the effect of the CEBP-binding site on promoter activity, we constructed vectors containing mutations of this site in the −442/+70 bp region dramatically reduced relative luciferase activity in ESCs compared with that of the wild-type (Fig. 5e). Taken together, these results suggest that CEBP positively regulates transcription of cNANO\textsubscript{G} by directly binding to the 5′ upstream promoter region in ESCs.

\textbf{Effects of predicted TFs on cNANO\textsubscript{G} gene transcription}

To confirm that the predicted TFs are expressed in PGCs and ESCs, we conducted RT-qPCR using RNA prepared from PGCs, ESCs, DF-1 cells, and CEFs. Expression of chicken CEB\textsubscript{P} genes (CEBP\textsubscript{A}, CEBP\textsubscript{B}, CEBP\textsubscript{D}, CEBP\textsubscript{G}, and CEBP\textsubscript{Z}) was significantly higher in ESCs than in other cells. By contrast, expression of
POU5F3 and SOX2/3 was significantly higher in PGCs and ESCs than in DF-1 cells and CEFs. Expression of POU5F3 and SOX3 did not differ between PGCs and ESCs, while SOX2 was significantly upregulated in PGCs. Additionally, expression of TP53 was significantly higher in PGCs than in other cells (Fig. 6).

We further examined whether these TFs affect the transcription of cNANOG in PGCs and ESCs using a siRNA-mediated knockdown assay. Knockdown of TP53 significantly increased cNANOG expression in PGCs, indicating that TP53 decreases cNANOG transcription (Fig. 7a). Knockdown of CEBPA, CEBPB, CEBPD, CEBPG, and CEBPZ significantly decreased cNANOG gene expression in ESCs (Fig. 7b–f). We also examined the luciferase activities driven by cNANOG promoter containing wild type binding sites after the knockdown of predicted TFs in PGCs and ESCs (Fig. 8). Knockdown of POU5F3 and SOX2 significantly reduced the activity of the cNANOG promoter fragment (−130/+70 bp) containing wild type binding sites, whereas, knockdown of TP53 is significantly increased the activity of the cNANOG promoter −250/+70 bp fragment in PGCs (Fig. 8a and b). Knockdown of CEBPA, CEBPB, CEBPD, CEBPG, and CEBPZ in ESCs dramatically reduced the activity of the cNANOG promoter −442/+70 bp fragment containing wild type CEBP binding site (Fig. 8c). These results indicate that these TFs control transcription of cNANOG by directly interacting with its promoter in a cell type-specific manner.

Discussion

The homeodomain TF NANO5 is important to maintain pluripotency in mammalian pluripotent cells during embryonic development [17]. Therefore, many studies have been conducted to determine how NANO5 expression is regulated by core factors in mammalian stem cells [20, 22, 23]. In addition, its expression is required for the formation of germ cells [30] and maintained in proliferating PGCs during the migration [42]. It has been recently reported that regulatory elements of NANO5 transcription in PGCs are different from the ES cells in mice but key regulatory factors have not yet been identified [43]. In chicken, NANO5 was also important for maintaining the pluripotency in PGCs and ESCs [31, 35, 38, 44]. However, the molecular mechanisms that regulate transcription of the NANO5 gene in chicken PGCs and ESCs remain unclear. In this regard, we characterized the structure of cNANOG and analyzed its promoter activity in chicken PGCs and ESCs.

We successfully transcribed cNANOG under the control of the proximal regulatory region located within 130 bp upstream of the TSS in PGCs. Furthermore, we
identified the regulatory region of cNANOG located within 442 bp upstream of the TSS in ESCs. Moreover, we showed that TP53 suppresses cNANOG transcription in PGCs. These results suggest that the cNANOG promoter functions in a cell type-specific manner. Similarly, Yeom et al. reported that the mouse Oct4 gene contains two separate regulatory elements [45]. The distal regulatory element is specifically active in mouse ESCs and EGCs, while the proximal enhancer is active in the epiblast. Thus, transcription of the mouse Oct4 gene is regulated in a stage-specific manner. Our findings indicate which elements are critical for gene expression in PGCs. This is the first report of a transcriptional regulatory factors of NANOG that is differentially active in a cell type-specific manner in chicken.

Many researchers have studied mammalian ESCs to determine which core factors regulate the NANOG gene. Most of the positive regulation of NANOG transcription has been discovered in the proximal region, which encompasses OCT3/4 and SOX2 in mouse ESCs. This region is strongly conserved in various mammalian species [20, 23]. Mutation of Octamer- and Sox-binding sites dramatically reduces transcription of NANOG. Therefore, OCT3/4 and SOX2 play an important role in regulation of the NANOG gene promoter in mammalian ESCs [23]. Also, these TFs such as POLISF3, SOX2/3, KL2, and SALL4 are highly expressed in chicken ES cells and PGCs [46]. According to the comparison of genomic sequence elements, core pluripotency factors of the mouse are not conserved with chicken [47]. In the present study, mutation of POLISF3- and SOX2-binding sites in the proximal region significantly reduced cNANOG promoter activity in PGCs. Although the DNA sequences of POLISF3 and SOX2, which are recognized by mouse core pluripotency factors, are not well conserved in chicken, POLISF3 and SOX2 are key regulators of cNANOG transcription. Further investigation by the electrophoretic mobility shift assay and chromatin immunoprecipitation sequencing is required to determine the core TFs in chicken PGCs.

Programmed death of PGCs is essential to remove abnormal, misplaced, and excess cells during PGC development and this is important to establish the next generation. In Drosophila melanogaster, TP53 is reportedly involved in elimination of excess PGCs during PGC development [48] and, mouse PGCs are regulated by p53.
to process the PGCs apoptosis [49]. In addition, TP53 binds to the NANOG promoter and suppresses NANOG expression for maintenance of genome stability in ESCs [28]. Interestingly, our results showed that the TP53-binding site negatively controlled NANOG transcription in chicken PGCs. Therefore, we propose that TP53 plays important roles in the regulation of NANOG transcription to maintain genome stability in PGCs.

CEBPB interacts with p300 to modulate histone acetylation [50], and p300 is a co-activator that binds to NANOG for maintenance of pluripotency in ESCs [51]. In our study, CEBPA, CEBPB, CEBPD, CEBPG, and CEBPZ were significantly upregulated in chicken ESCs. In addition, knockdown of these TFs dramatically decreased transcription of cNANOG in chicken ESCs. These results suggest that CEBP in chicken ESCs participate in regulation of cNANOG transcription by directly interacting with putative binding sites in the cNANOG promoter.

As described above, transcription regulation of cNANOG is conserved in mammals, although DNA sequences of regulation factors differ between chicken and mammals. Typically, mammalian PGCs can be induced by cell signaling [52]. Interestingly, mouse Nanog is key regulator of PGCs-like cells independent of BMP4 and Wnt signals by activating the expression of germ cell-

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Fig. 8 Chicken NANOG promoter activity after knockdown of predicted transcription factors. a Luciferase activity of pNL-NANOG-130/+ 70 after the knockdown of POUF3 and SOX2 in chicken primordial germ cells (PGCs). b Luciferase activity of pNL-NANOG – 250/+ 70 after the knockdown of TP53 in chicken PGCs. c Luciferase activity of pNL-NANOG – 442/+ 70 after the knockdown of CEBPA, CEBPB, CEBPD, CEBPG, and CEBPZ in chicken embryonic stem cells (ESCs). Error bars indicate the standard deviation of triplicate analyses. Significant differences are indicated as ns, no significance, * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001
specific TFs [33]. On the other hand, chicken germ cells may be specified by maternally inherited factors like VASA and DAZL in germ plasm [53, 54]. Recently, the epigenetic regulation of NANO/G in chicken PGCs has been investigated by our group to understand the molecular mechanisms involved in the specification of germ cells [34]. However, the regulation of cNANOG in chicken germ cell specification is still unclear. In this study, we shown that chicken NANO/G has differential regulatory roles in PGCs and ESCs, even though cNANO/G promoter region sharing the common transcription factor binding sites. These finding provided insights into germ cell and stem cell-specific transcriptional regulatory mechanisms.

**Conclusion**
This study demonstrated that the proximal regulatory region of the cNANO/G gene differs between PGCs and ESCs. We showed that the cNANO/G gene is positively regulated by POLISF3 and SOX2 and negatively regulated by TP53 in PGCs, while it is positively regulated by CEBP in ESCs. Collectively, these findings aid understanding of transcriptional regulation of the cNANO/G gene in PGCs and ESCs (Fig. 9).

**Abbreviations**
TF: Transcription factor; ESCs: Embryonic stem cells; PGCs: Primordial germ cells; cNANO/G: Chicken NANO/G; CEBS: Embryonic germ cells; TSS: Transcription start site; HHS: Hamburger and Hamilton stage 5; WL: White Leghorn; 5′-RACE: 5′ Rapid amplification of cDNA ends; GSPs: Gene-specific primers; Fluc: Firefly luciferase; Nluc: NanoLuc luciferase; AU: Arbitrary units; CEF: Chicken embryonic fibroblasts; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

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**Authors’ contributions**
HJC participated in the design of the study, carried out the experiments, statistical analysis and wrote the first draft of the manuscript. SDJ, JHK, and DR carried out and analyzed the experiments. DR, BP, JYH participated in writing the final versions of the manuscript. JYH participated in the design of the study and overall coordination. All authors have read and approved the final manuscript.

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**Availability of data and materials**
The datasets during and/or analyzed during the current study available from the corresponding authors on reasonable request.

**Ethics approval and consent to participate**
The care and experimental use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University.
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