Differential Transcriptional Regulation of the NANOG Gene in Chicken Primordial Germ Cells and Embryonic Stem Cells

Hee Jung Choi
Seoul National University College of Agriculture and Life Sciences
So Dam Jin
Seoul National University College of Agriculture and Life Sciences
Deivendran Rengaraj
Seoul National University College of Agriculture and Life Sciences
Jin Hwa Kim
Seoul National University College of Agriculture and Life Sciences
Bertrand Pain
INSERM U1208: Cellule Souche et Cerveau
Jae Yong Han (✉ jaehan@snu.ac.kr)
Seoul National University College of Agriculture and Life Sciences https://orcid.org/0000-0003-3413-3277

Research

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Abstract

Background

NANOG is a core transcription factor (TF) in embryonic stem cells (ESCs) and primordial germ cells (PGCs). Regulation of the *NANOG* gene by TFs, epigenetic factors, and autoregulatory factors is well characterized in ESCs, and transcriptional regulation of *NANOG* is well established in these cells. Although *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 *NANOG* (*cNANOG)* gene in PGCs and ESCs.

Results

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

Conclusions

We show for the first time that different *cis*-regulatory elements control transcription of *cNANOG* in a cell type-specific manner. This finding might help to elucidate the mechanism that regulates *cNANOG* expression in PGCs and ESCs.

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). In addition, TFs generally initiate and guide cell fate such as lineage progression and control the stability of cell differentiation (3). 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 (4). 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 transcription (5). Also, germ cells reorganize different type of core promoter TFs under the control of germ cell-specific TFs during germ cell differentiation (6).

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) (7-9). In particular, the *NANOG* gene is important for acquisition of pluripotency by ESCs and embryonic germ cells (EGCs) (10-12). Several earlier studies identified the regulatory elements of *NANOG* that are required to maintain the self-renewal and pluripotency of ESCs (13-15). 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 (13, 16). Direct binding of *ZFP143* to the proximal region of the *NANOG* promoter regulates *NANOG* expression by modulating *OCT4* binding (17). In addition, TF-binding *cis*-regulatory elements of *NANOG*, including *SP1/SP3*-, *SALL4*-, and *BRD4*-binding sites, have been identified as positive regulators (18-20). On the other hand, *P53*-binding sites negatively regulate *NANOG* expression to induce differentiation of ESCs (21). 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, POU5F3*, and *SOX2*, and their expression controls transcription of germness-related genes in these cells (6, 22). During early germ cell development, *NANOG* plays an essential role as a key TF required for formation of PGCs and maintenance of early germ cells (23, 24). *NANOG*-deficient PGCs reportedly undergo apoptotic death (25). It was recently reported that *NANOG* regulates PGC-specific epigenetic programming and global histone methylation (26, 27). *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 (28-30). Similar to mammals, *NANOG* is crucial to maintain pluripotency and self-renewal of chicken ESCs (28). *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 (24, 28, 31).

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 *cis*-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.

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.

Culture of chicken PGCs, ESCs, and DF-1 cells

WL PGCs were maintained and sub-passaged in KnockOut DMEM (Thermo Fisher-Invitrogen, USA) supplemented with 20% fetal bovine serum (Hyclone, South Logan, UT, USA), 2% chicken serum (MilliporeSigma, Burlington, MA, USA), 1× nucleosides (MilliporeSigma), 2 mM L-glutamine, 1× nonessential amino acids, β-mercaptoethanol, 10 mM sodium pyruvate, 1× antibiotic-antimycotic (ABAM; Thermo Fisher-Invitrogen), and 10 ng/mL human basic fibroblast growth factor (MilliporeSigma). PGCs were sub-cultured onto mitomycin-inactivated mouse embryonic fibroblasts at an interval of 5–6 d via gentle pipetting.

Chicken ESCs were generously provided by Dr. Bertrand Pain (INSERM-INRAE). These cells were maintained and sub-passaged as previously described (32). Briefly, ESCs were cultured in 50 mL of DMEM/F12 (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone), 1× nonessential amino acids, 10 mM sodium pyruvate, β-mercaptoethanol, 1× ABAM (Thermo Fisher-Invitrogen), 5 ng/mL insulin-like growth factor 1, 1 ng/mL stem cell factor, 1 ng/mL interleukin 6, 1 ng/mL soluble interleukin 6 receptor α, and 1,000 U/mL human leukemia inhibitory factor. ESCs were sub-cultured onto mitotically inactivated STO cells.

Chicken DF-1 cells (CRL-12203; American Type Culture Collection, USA) and chicken embryo fibroblasts (CEFs) were cultured as negative controls. Chicken DF-1 cells were maintained and sub-passaged in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 1× ABAM (Thermo Fisher-Invitrogen). CEFs were derived from 6-day-old WL embryos and maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 1× ABAM (Thermo Fisher-Invitrogen). All chicken cells (PGCs, ESCs, DF-1 cells, and CEFs) were cultured in an incubator at 37°C under an atmosphere of 5% CO_2 and 60–70% relative humidity.

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 ACG CCA 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 efficiency 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 mM dNTP mixture, and 10 pmol each of gene-specific forward and reverse primers (Table 3). RT-qPCR was performed in triplicate. Relative 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 −3,550/+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 −3,550/+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 (−3,550/+70 bp sequence) was similar between PGCs and ESCs but *cNANOG* transcription is differentially regulated in PGCs and ESCs by the proximal enhancer.

POU5F3 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). These results suggest that a positive transcriptional *cis*-regulatory element is located between −130 and −69 bp.

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*-binding sites, which contain binding sites for POU5F3 (*OCT4), SOX2, NANOG*, and *TCF3*, were identified in this region (Fig. 3b). Sequence alignment of this *cNANOG* promoter region from six vertebrate species showed that the *POU5F3*- and *SOX2*-binding *cis*-regulatory elements are highly conserved in mammalian species (Fig. 3c). 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. 3d). 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 alone than by mutation of the *POU5F3*-binding site alone in PGCs (Fig. 3e). Taken together, these results suggest that *POU5F3* 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 *cis*-regulatory element is located between −250 and −210 bp. To investigate 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 positive regulators of *NANOG* transcription (21, 33, 34). We further examined whether *TP53* affects *cNANOG* 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 *cNANOG* promoter region significantly increased luciferase activity in PGCs (Fig. 4c). These results demonstrate that *TP53* suppresses *cNANOG* transcription in PGCs.

CEBP transactivates the *cNANOG* promoter in ESCs

To further investigate the potential transcriptional *cis*-regulatory elements in ESCs, we generated four constructs harboring fragments of the −442/+70 bp region of the *cNANOG* 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 *cNANOG* promoter activity in ESCs (Fig. 5a). These results suggest that a positive transcriptional *cis*-regulatory element is located between −442 and −407 bp.

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 *cNANOG* gene in ESCs. Only a *CEBP*-binding site was identified between −442 and −407 bp (Fig. 5b). To examine the effect of the *CEBP*-binding site on promoter activity, we constructed vectors containing mutations of this site in the −422/+70 bp fragment (Fig. 5c). Mutation of the *CEBP*-binding site in the −442/+70 bp region dramatically reduced relative luciferase activity in ESCs compared with the wild-type construct of the same region (Fig. 5d). Taken together, these results suggest that *CEBP* positively regulates transcription of *cNANOG* by directly binding to the 5’ upstream promoter region in ESCs.

Effects of predicted TFs on *cNANOG* 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 *CEBP* genes (*CEBPA, CEBPB, CEBPD, CEBPG*, and *CEBPZ*) 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 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*, and *CEBPG* significantly decreased *cNANOG* gene expression in ESCs (Fig. 7b–f). 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 NANOG is important to maintain proliferation of germ cells and pluripotency of stem cells (35). However, the molecular mechanisms that regulate transcription of the *NANOG* gene in chicken PGCs and ESCs remain unclear, although *cNANOG* is evolutionarily conserved with *NANOG* orthologs from zebrafish and axolotl (28-30). 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 (36). 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 *cis*-regulatory element 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. To understand the molecular networks, several TFs, including *OCT3/4, SOX2, SALL4, PBX1,* and *KLF4*, have been identified in mouse, human, and goat (13, 19, 37, 38). *OCT3/4* and *SOX2* play an important role in regulation of the *NANOG* gene promoter. Mutation of Octamer- and Sox-binding sites dramatically reduces transcription of *NANOG*. Also, these TFs such as *POU5F3, SOX2/3, KLF2, and SALL4* are highly expressed in chicken ES cells and PGCs (39). In the present study, mutation of *POU5F3*- and *SOX2*-binding sites in the proximal region significantly reduced c*NANOG* promoter activity in PGCs. Although the DNA sequences of *POU5F3* and *SOX2,* which are recognized by mouse core pluripotency factors, are not well conserved in chicken, *POU5F3* and *SOX2* are key regulators of c*NANOG* 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. *TP53* is reportedly involved in elimination of excess PGCs during PGC development (40). In addition, *TP53* binds to the *NANOG* promoter and suppresses *NANOG* expression for maintenance of genome stability in ESCs (21). Our results showed that the *TP53*-binding site negatively controlled *NANOG* transcription in 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 (41), and *p300* is a co-activator that binds to *NANOG* for maintenance of pluripotency in ESCs (42). 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.

We found that specific TFs were important in different cell types. This suggests that the notion of cell type-specific TFs based on expression analysis should be carefully considered, especially in the context of transcriptional regulation. In addition, analysis of cell type-specific *cis*-regulatory elements indicated that TFs control *NANOG* expression for cell type-specific functions. Thus, given that cell type-specific functions must be regulated, it is logical that differences in complex regulation of TFs underlie transcriptional regulation specific to each cell type.

Conclusion

This study demonstrated that the proximal regulatory region of the *cNANOG* gene differs between PGCs and ESCs. We showed that the *cNANOG* gene is positively regulated by *POU5F3* 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 *cNANOG* gene in PGCs and ESCs (Fig. 8).

Abbreviations

TF: transcription factor; ESCs: embryonic stem cells; PGCs: primordial germ cells; cNANOG: chicken NANOG; CEBP: CCAAT/enhancer-binding protein; EGCs: embryonic germ cells; TSS: transcription start site; HH5: 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 embryo fibroblasts; GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Declarations

Ethics approval

The care and experimental use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analyzed during the current study available from the corresponding authors on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JYH participated in study design and coordination. 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. BP participated in writing the final versions of the manuscript. All authors have read and approved the final manuscript.

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Author details

[1]Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea. [2]Univ Lyon, Universite ́Lyon 1, INSERM, INRAE, Stem Cell and Brain Research Institute, U1208, USC1361, 69500 Bron, France. [3]Institute for Biomedical Sciences, Shinshu University, Minamiminowa, Nagano 399-4598, Japan.

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Tables

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

Primer name	Primer sequence (5' ⟶ 3')
cNANOG -3,550 bp_F	AAGCTTTGTCCTTTTCTTGACC
cNANOG -3,375 bp_F	CTGGAGTCAAGGGCTGTGG
cNANOG -3,154 bp_F	TGGGCCCCTCGTTACAGCT
cNANOG -2,928 bp_F	CCAGCAGTACAAGCTCCGAA
cNANOG -1988 bp_F	GCGACACGTGGAACA
cNANOG -945bp_F	CATGGGGGTGTCTGCTC
cNANOG -627 bp_F	CTTCTTTGTGCTCCTCC
cNANOG -442 bp_F	CTGCAGTCTGCAATGC
cNANOG -407 bp_F	AATGTCCCGGGGGGGTCTCTGG
cNANOG -377 bp_F	CCATTCTTTGTACTTGGGTGGGGACCGATGAG
cNANOG -312 bp_F	CGAGGGCGGGGGTGCCAGCCCAG
cNANOG -250 bp_F	CTGCAGTCTGCTCCTCC
cNANOG -210 bp_F	CTGCAGTCTGCAATGC
cNANOG -170 bp_F	CCAAAGGGGGAAGCTGC
cNANOG -130 bp_F	ACTCTCCGAATATCCCCATAGC
cNANOG -69 bp_F	TCGTGACAATCTCTTG
cNANOG promoter_R	GGTCGGGACGACACCT

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

Target gene	siRNA sequence (5' ⟶ 3')	
	Sense	Antisense
TP53	UCAUGGACCUCUGGAGCAU	AUGCUCCAGAGGUCCAUGA
CEBPA	GCGAGGAGGAGGAGGUGA	UUCACCUCCUCCUCCUCGC
CEBPB	GCGCAAGAGCCGCGACAAA	UUUGUCGCGGCUCUUGCGC
CEBPD	ACGAGAAGCUGCACAAGAA	UUCUUGUGCAGCUUCUCGU
CEBPG	AAAUUAAGCUCCUGACCAA	UUGGUCAGGAGCUUAAUUU
CEBPZ	GAGAAAAGCAAGAAGGAAA	UUUCCUUCUUGCUUUUCUC

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

Gene symbol	Primer sequence (5′ ⟶ 3′)	
	Forward	Reverse
CEBPA	CCCACCTGCAGTACCAGATC	TCTTTTTGGATTTGCCGCGG
CEBPB	CGCCCGCCTTTAAATCCATG	GGGCTGAAGTCAATGGCTCT
CEBPD	ACTTCTACGACGCCAAGGTG	CTCTCGTCCTCGTACATGGC
CEBPG	CCCACAGCTAACGTGTCAGT	GGACGGGCTCTTCTTTGACA
CEBPZ	CGCTGTTCACAGTCTCCACT	GGACGCTGTGAGAAAGACCA
SOX2	AAACCGAGCTGAAACCTCCC	TGTGCATCTTCGGGTTCTCC
SOX3	CGGCTCAGCAGACTCGATAC	TCGCCGTGGCTTAAGAACTT
POUV	TGAAGGGAACGCTGGAGAGC	ATGTCACTGGGATGGGCAGAC
TP53	CCGTGGCCGTCTATAAGAAA	ACAGCACCGTGGTACAGTCA
NANOG	AGTGGCAGAGTCTGGGGTAT	ACTACTACTGGCCCTCTCCG
GAPDH	GGTGGTGCTAAGCGTGTTAT	ACCTCTGTCATCTCTCCACA

Figures

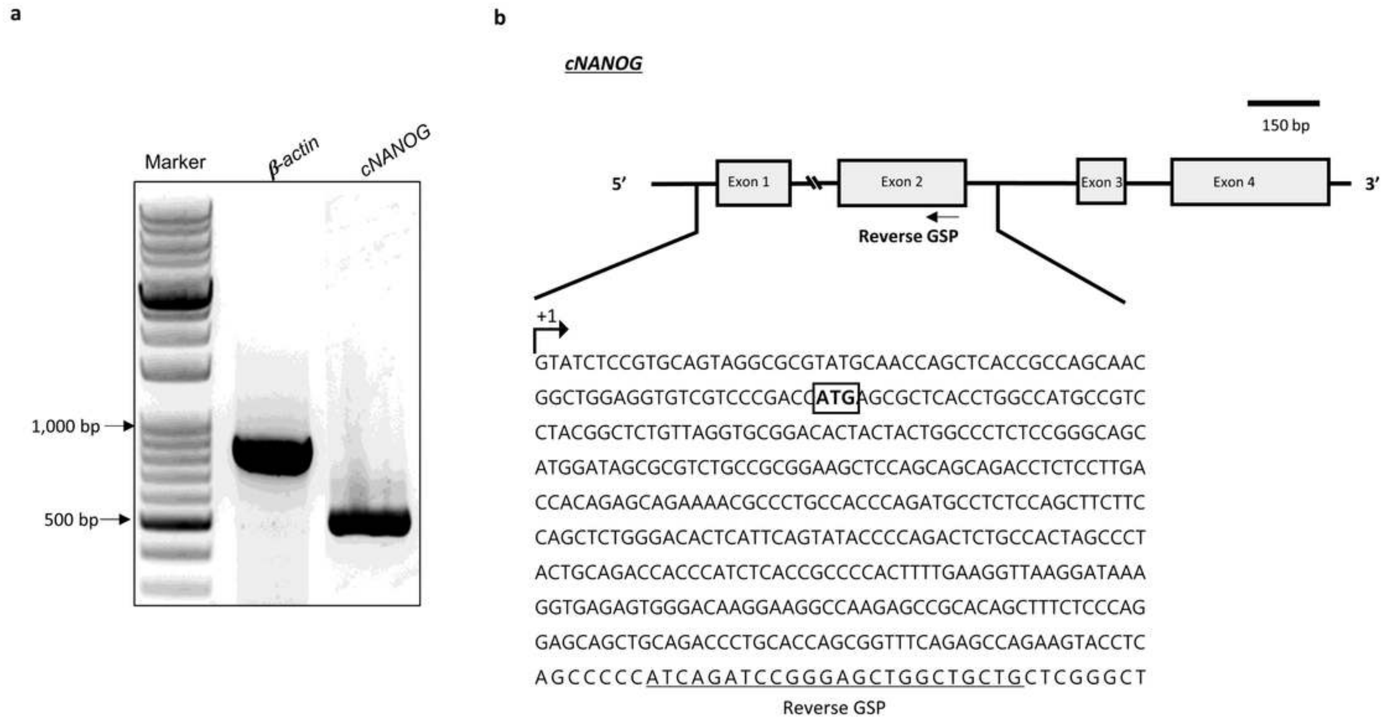

Figure 1

Identification of the transcription start site (TSS) of the chicken NANOG (cNANOG) gene by 5’-rapid amplification of cDNA ends (RACE) analysis. a After performing 5’-RACE, the PCR product was analyzed and its size was determined by agarose gel electrophoresis. Scale bar = 150 bp. b The 5’-RACE product was cloned into the pGEM-T vector and sequenced. The TSS of the cNANOG gene is located 70 bp upstream of the translation start codon ATG. +1 indicates the potential TSS of the cNANOG gene.

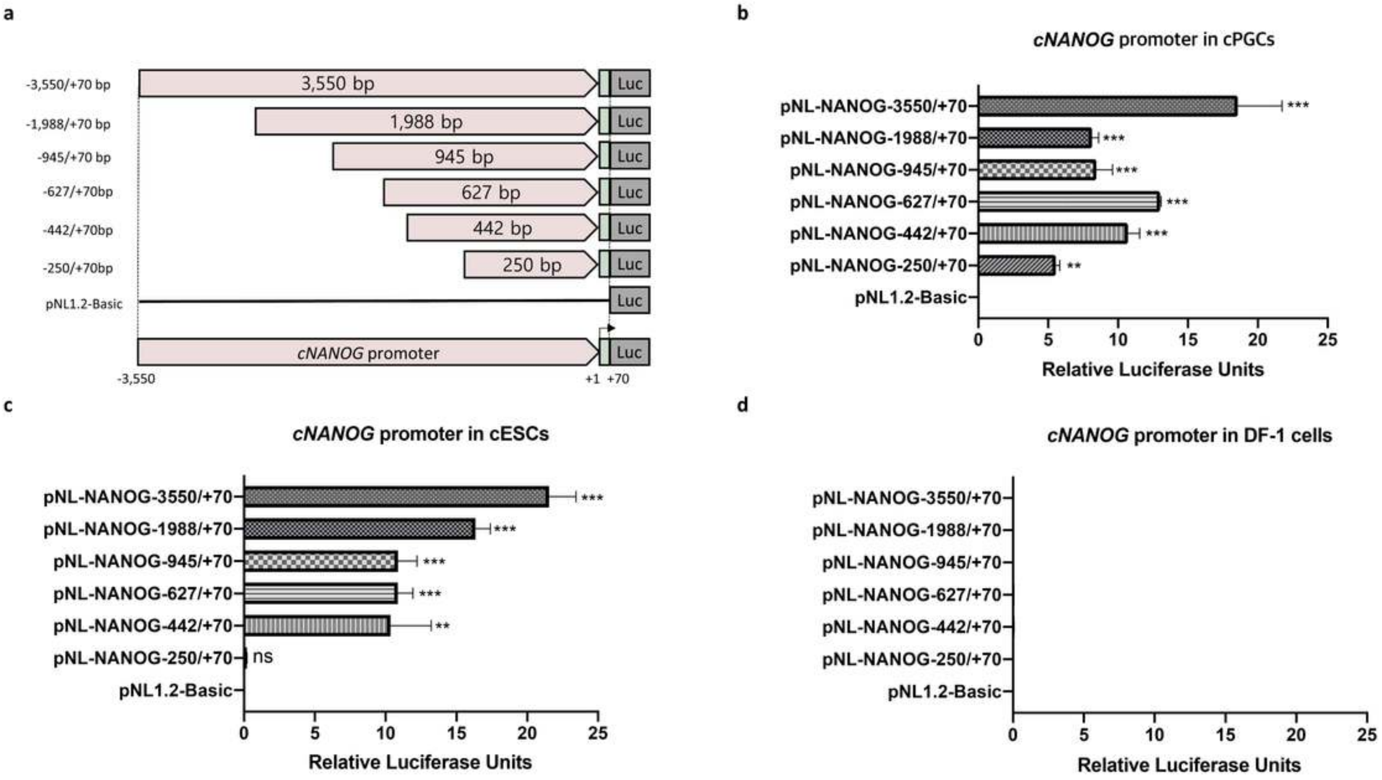

Figure 2

Promoter variants reduce activity of the chicken NANOG (cNANOG) gene in a cell type-dependent manner. a Schematic diagram of deletion of the cNANOG gene promoter (−3,550/+70 bp). Relative luciferase activity in chicken primordial germ cells (PGCs) (b), chicken embryonic stem cells (ESCs) (c), and DF-1 cells (d). Luciferase activity was normalized against firefly luciferase expression (pNL1.2-Basic) to control for variation in the transfection efficiency. Significant differences are indicated as ns (no significance), ** $P < 0.01$, and *** $P < 0.001$.

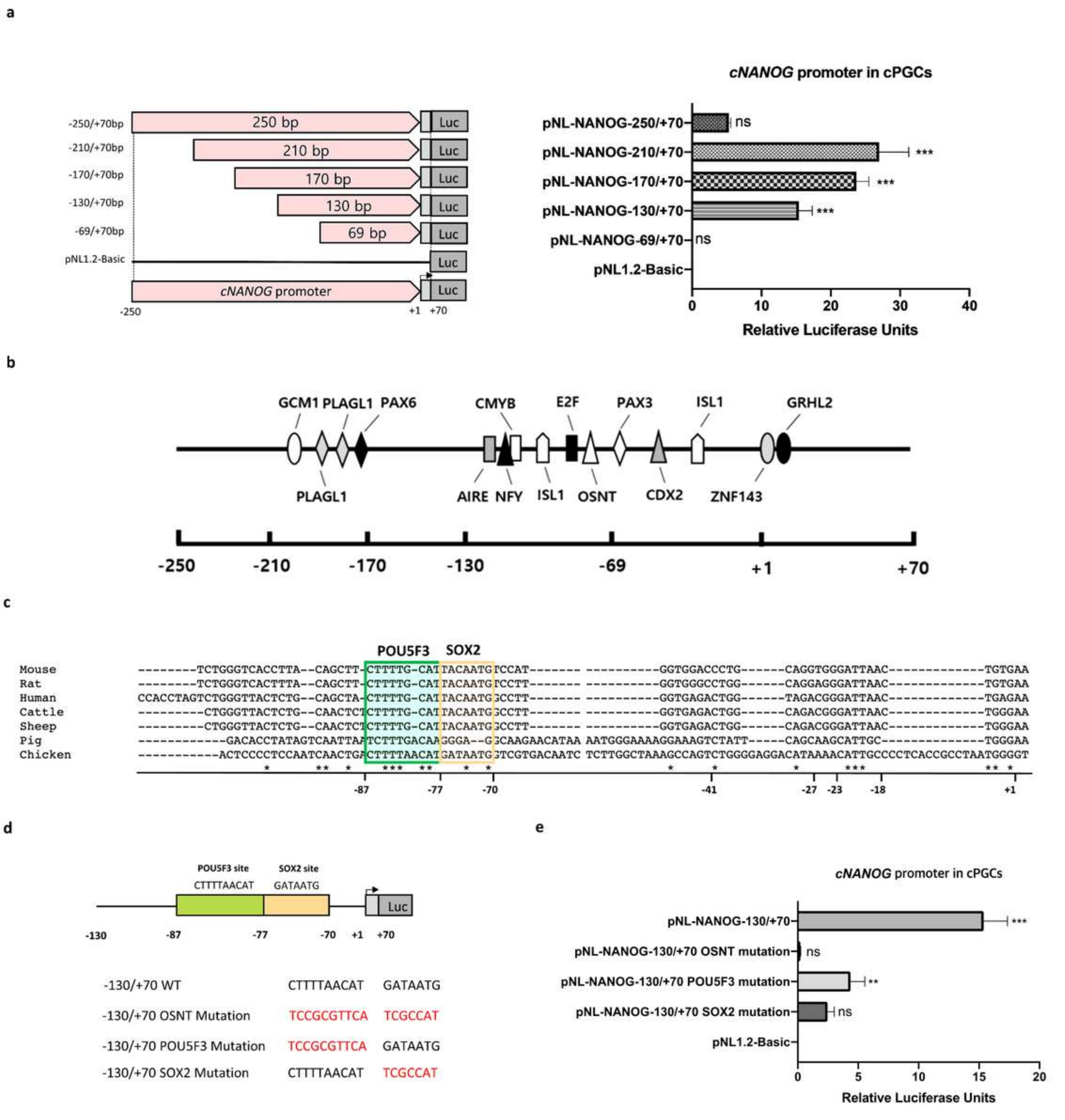

Figure 3

Verification of the proximal enhancer of the chicken NANOG (cNANOG gene) in chicken primordial germ cells (PGCs). a Schematic diagram of the constructed cNANOG promoter vectors and luciferase activity. b Prediction of transcription factor (TF)-binding sites in the cNANOG promoter region located from −250 to +70 bp. c Multiple alignment of the putative cNANOG proximal enhancer with cis-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. d Mutation analysis of putative POU5F3- and SOX2-binding sites in PGCs. e 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.

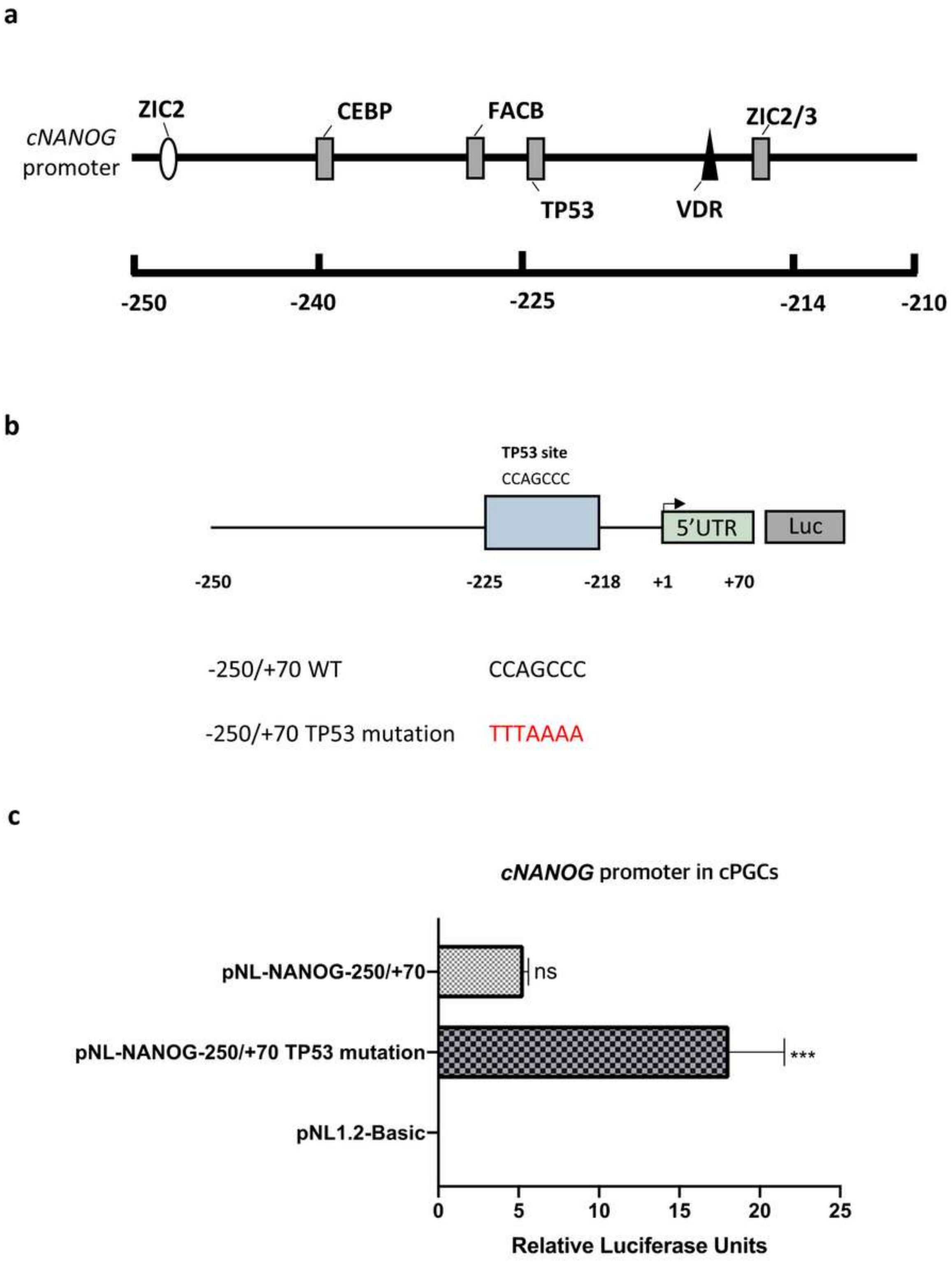

Figure 4

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 Mutation TP53) vectors. pNL1.2-Basic was used as a control. Significant differences are indicated as ns (no significance) and *** P < 0.001.

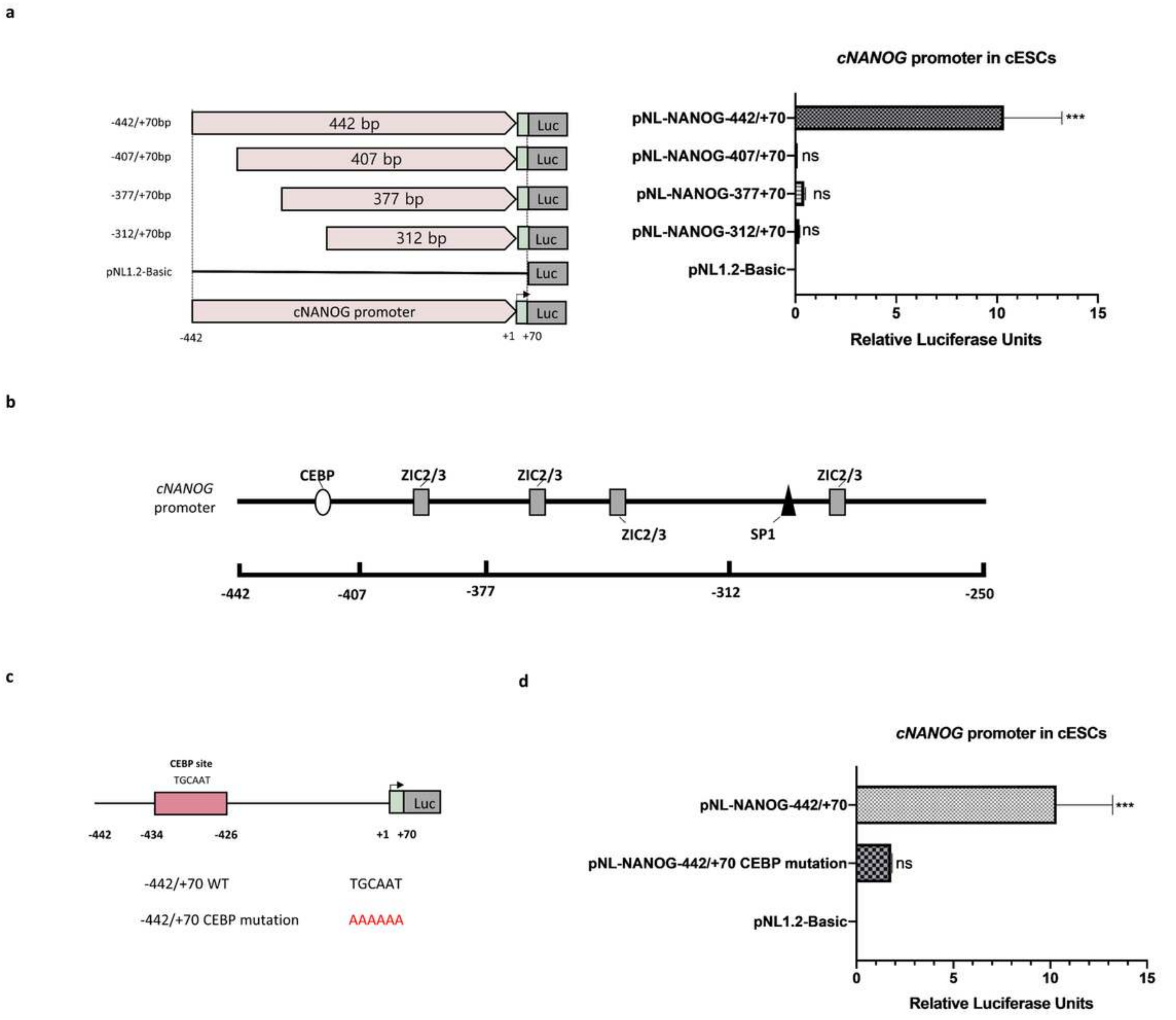

Figure 5

Verification of the proximal enhancer of the chicken NANOG (cNANOG) gene in chicken embryonic stem cells (ESCs). a Schematic diagram of the constructed cNANOG promoter vectors and luciferase activity. b Prediction of transcription factor (TF)-binding sites in the cNANOG promoter region from −442 to −250 bp. c Mutation analysis of putative CCAAT/enhancer-binding protein (CEBP)-binding sites in ESCs. d Luciferase activity of the −442/+70 bp cNANOG promoter fragment compared with that of the mutated promoter. Significant differences are indicated as ns (no significance) and *** P < 0.001.

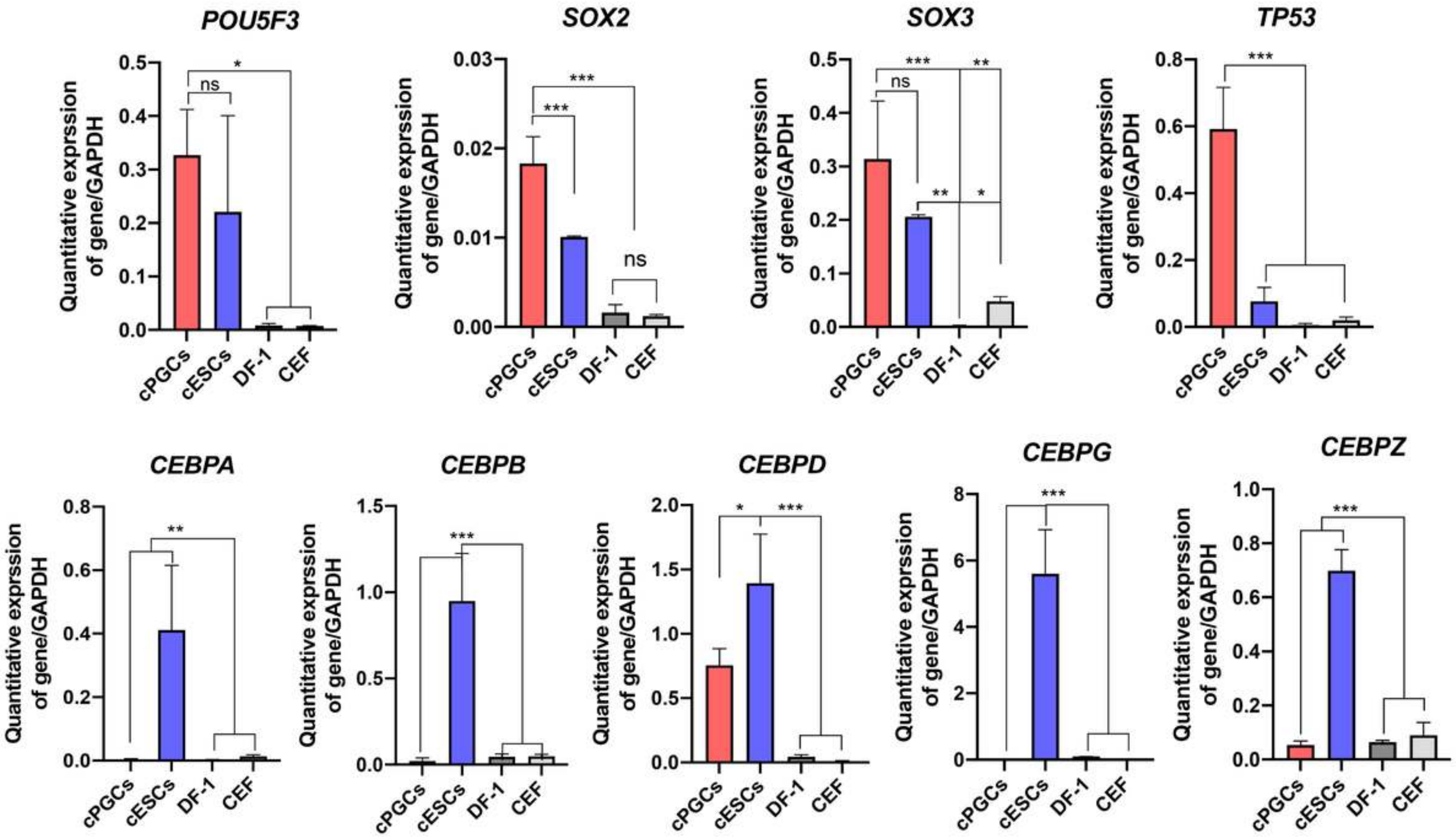

Figure 6

Quantitative expression analysis of predicted transcription factors (TFs) in various cell types. Expression of predicted TFs in chicken primordial germ cells (PGCs), embryonic stem cells (ESCs), DF-1 cells, and chicken embryonic fibroblasts (CEFs) was analyzed by quantitative RT-PCR. Error bars indicate the standard deviation of triplicate analysis. Significant differences are indicated as ns (no significance), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

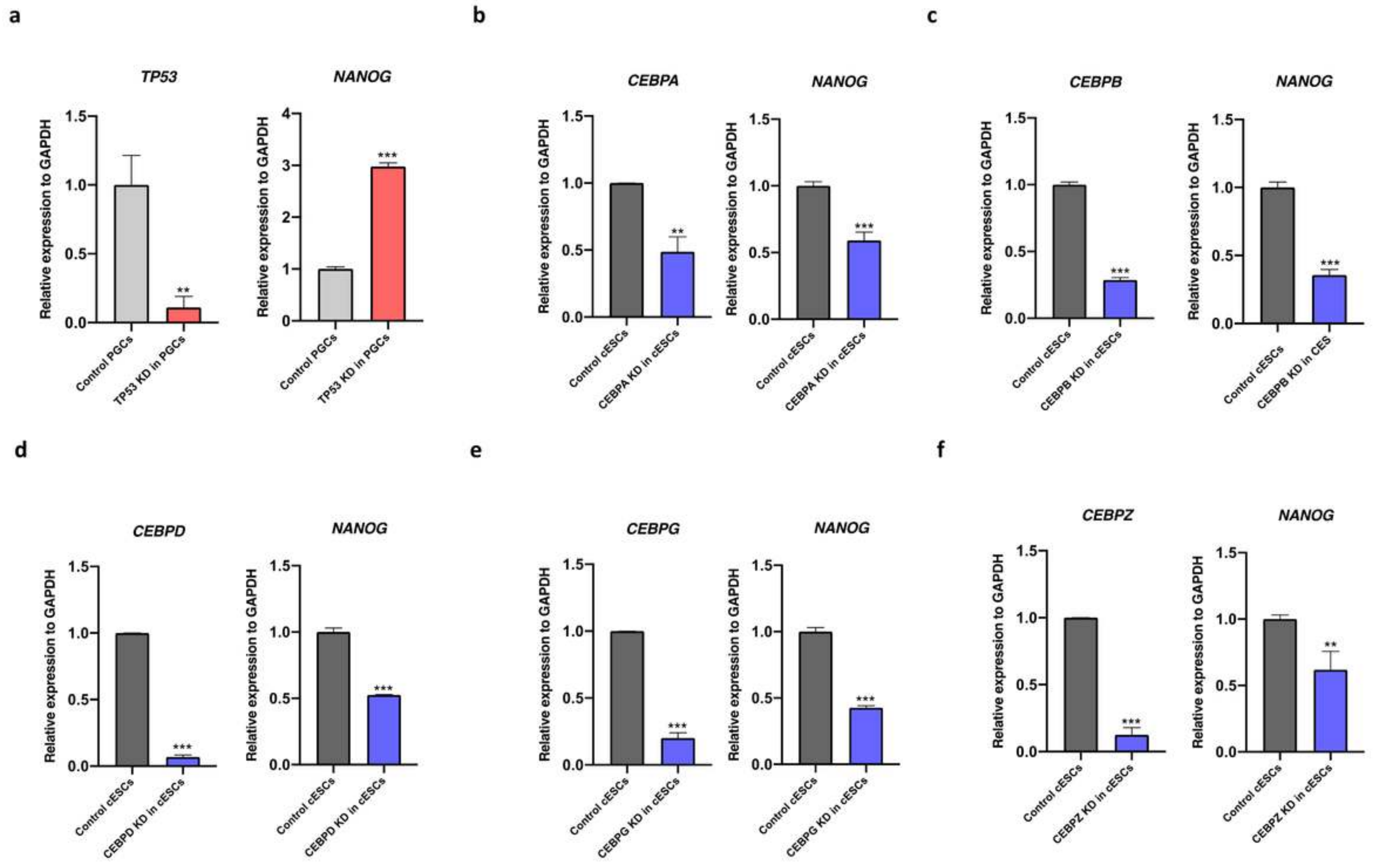

Figure 7

Relative gene expression analysis after knockdown of predicted transcription factors (TFs) in cultured primordial germ cells (PGCs) and embryonic stem cells (ESCs). a Efficiency of small interfering RNA (siRNA)-mediated knockdown of TP53 in PGCs was analyzed by quantitative reverse-transcription PCR (RT-qPCR). Relative expression of NANOG was determined. b−f Efficiency of siRNA-mediated knockdown of CEBPA, CEBPB, CEBPD, CEBPG, and CEBPZ in ESCs was analyzed by RT-qPCR. Relative expression of NANOG was determined in each sample. Error bars indicate the standard deviation of triplicate analyses. Significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

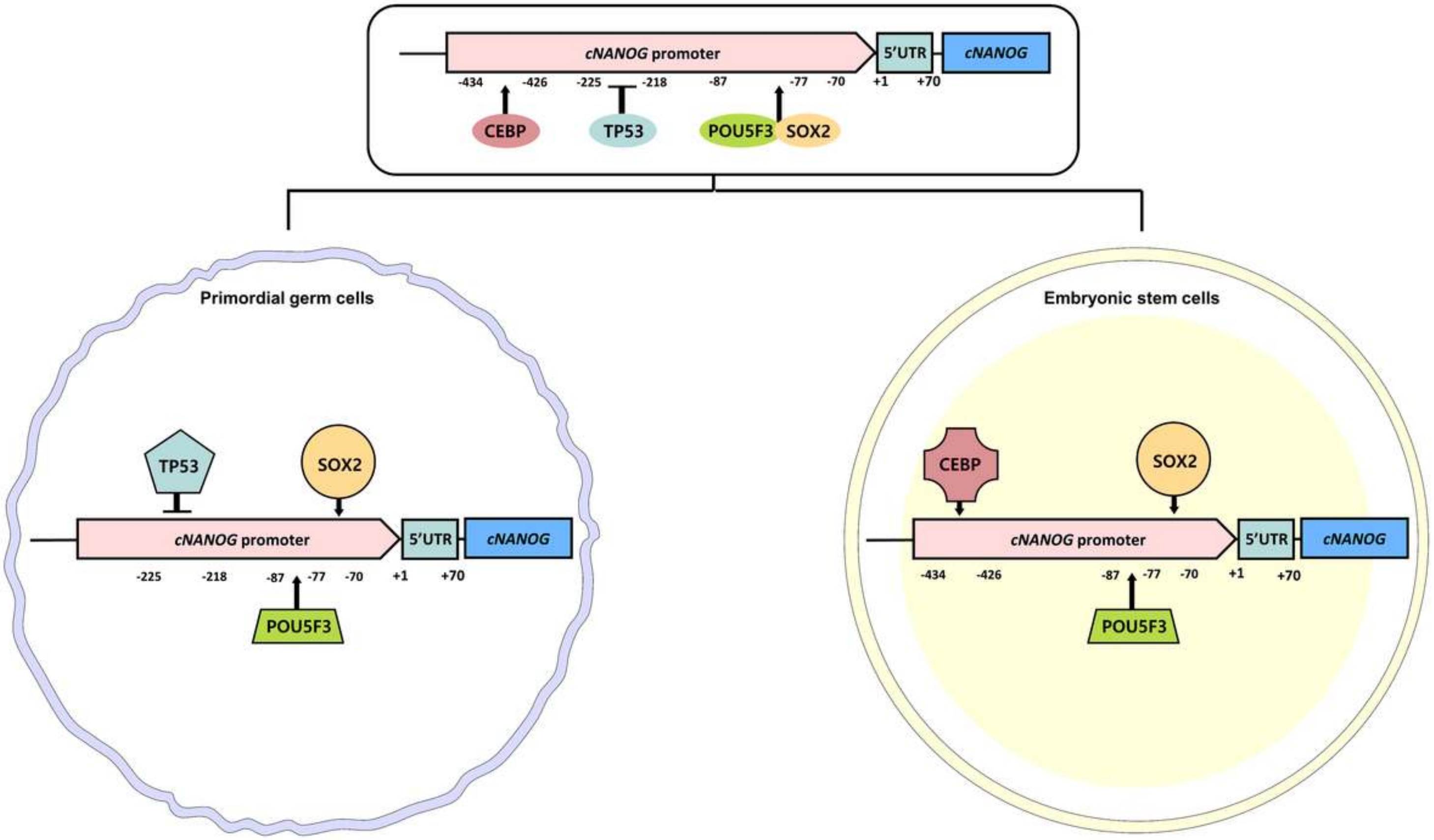

Figure 8

A model illustrating regulation of chicken NANOG (cNANOG) gene transcription in chicken primordial germ cells (PGCs) and embryonic stem cells (ESCs). cNANOG gene expression requires transcriptional cis-regulatory elements that are positively controlled by POU5F3 and SOX2 and negatively controlled by TP53 in PGCs. On the other hand, CCAAT/enhancer-binding protein (CEBP) positively regulates cNANOG gene expression in ESCs.