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Specific Knockdown of Nanog Expression by RNA Interference in P19 Embryonal Carcinoma Stem Cells

Seung-Cheol Choi and Do-Sun Lim†

Dept. of Cardiology, College of Medicine, Korea University, Seoul 136-705, Korea

P19 배아 암종 줄기세포에서 RNA 간섭에 의한 Nanog 유전자 발현의 특이적 억제

최 승 철 · 임 도 선†

고려대학교 의과대학 순환기내과

ABSTRACT : Nanog is a newly identified member of the homeobox family of DNA binding transcription factors that functions to maintain the undifferentiated state of stem cells. However, molecular mechanisms underlying the function of Nanog remain largely unknown. To elucidate the regulatory roles of Nanog involved in maintenance of P19 embryonal carcinoma (EC) stem cells, we transfected three small interfering RNA (siRNA) duplexes targeted against different regions of the Nanog gene into P19 cells. The Nanog siRNA-100 duplexes effectively decreased the expression of Nanog up to 30.7% compared to other two Nanog siRNAs, the Nanog siRNA-400 (67.9 %) and -793 (53.0%). When examined by RT-PCR and real-time PCR, the expression of markers for pluripotency such as Fgf4, Oct3/4, Rex1, Sox1 and Yes was downregulated at 48 h after transfection with Nanog siRNA-100. Furthermore, expression of the ectodermal markers, Fgf5 and Isl1 was reduced by Nanog knockdown. By contrast, the expression of other markers for pluripotency such as Cripto, Sox2 and Zfp57 was not affected by Nanog knockdown at this time. On the other hand, the expression of Lif/Stat3 pathway molecules and of the endoderm markers including Dab2, Gata4, Gata6 and the germ cell nuclear factor was not changed by Nanog knockdown. The results of this study demonstrated that the knockdown of Nanog expression by RNA interference in P19 cells was sufficient to modulate the expression of pluripotent markers involved in the self-renewal of EC stem cells. These results provide the valuable information on potential downstream targets of Nanog and add to our understanding of the function of Nanog in P19 EC stem cells.

Key words : Nanog, RNA interference, siRNA, P19 embryonal carcinoma stem cells.

INTRODUCTION

The P19 embryonal carcinoma (EC) stem cell line is derived from a teratocarcinoma formed in C3H/He mice (McBurney et al., 1982). This cell line has the ability to differentiate into all three germ layers (McBurney et al., 1982). In addition, they maintain the undifferentiated state in a monolayer, without a feeder-cell layer, which allows for the easy introduction of ectopic genes. Due to their advantages over embryonic stem (ES) cells, therefore, P19 cells have been used to study molecular pathways that are involved in self-renewal as well as differentiation (Qin et al., 2004; Hough et al., 2006).

Introduction of double-stranded RNA (dsRNA) into *Drosophila* embryos or adult *Caenorhabditis elegans* induces sequence-specific posttranscriptional gene silencing known as RNA interference (RNAi) (Fire et al., 1998; Kennerdall & Carthew, 1998). In mammalian cells, 20～23-nucleotide small interfering RNA (siRNA) duplexes specifically have been shown to specifically suppress the expression of

† Correspondence: Dept. of Cardiology, College of Medicine, Korea University, Seoul 136-705, Korea, Tel: +82-2-920-5445, Fax: +82-2-927-1478, E-mail: dslmd@kumc.or.kr

endogenous and heterologous genes in different mammalian cell lines (Elbashir et al., 2001). Currently, RNAi is widely used to silence expression of specific genes in ES cells and EC cells (Hyslop et al., 2005; Hough et al., 2006). It is well known that selection of highly active and specific siRNA sequences to target genes is critical for achieving efficient and specific gene silencing in RNAi experiments (Elbashir et al., 2002).

Nanog is a critical factor underlying pluripotency in both the inner cell mass (ICM) and ES cells (Chambers et al., 2003; Mitsui et al., 2003). Moreover, Nanog is needed to sustain the undifferentiated status of EC cells (Hyslop et al., 2005; Chen et al., 2006). Nanog-deficient ES cells loose pluripotency and differentiate into extraembryonic endoderm lineages (Mitsui et al., 2003). Indeed, Nanog directly repressed expression of Gata6, an extraembryonic endoderm marker through its binding to the promoter region of the Gata6 gene (Singh et al., 2007). The mechanism by which Nanog regulates pluripotency and inhibits primitive endoderm differentiation in ES cells is thought to be due to the formation of transcriptional networks with multiple transcription regulators including Oct3/4 and Sox2 (Boyer et al., 2005; Liang et al., 2008). Several lines of evidence have provided new insights into the regulatory mechanisms involved in the downstream targets of Nanog. Nanog expression was suppressed by p53 after binding to the promoter of Nanog, which resulted in the differentiation of mouse ES cells (Lin et al., 2004). Nanog maintained pluripotency of mouse ES cells by inhibiting NFκB while cooperating with STAT3 (Torres & Watt, 2008).

To date, however, the molecular mechanisms underlying the downstream targets of Nanog remain largely undefined. To elucidate the regulatory mechanisms of Nanog and identify the potential downstream targets of Nanog, we transiently reduced Nanog expression by transfection of the siRNAs targeted against the Nanog gene in P19 cells, and examined the expression of stem cell markers as well as the different cell lineage markers by both reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR in the Nanog siRNA-transfected P19 cells.

MATERIALS AND METHODS

1. Small Interfering RNA Synthesis

The sequences for siRNA against Nanog were selected using the siRNA Target Finder program at Ambion's Website (http://www.ambion.com). Twenty-one nucleotides within the target mRNA with an amino acid dinucleotide were selected with a GC content within the range of 30～50%. All siRNA sequences designed were subjected to a BLAST search; the sequences with less than 16 nucleotides of homology to other genes in the mouse genome were selected for the experiments. The nucleotides designed for the Nanog siRNA were as follows: 5'-AACTATTCTTGCTTACAAGGG-3' (GenBank accession number AY278951, 100-120, siRNA-100); 5'-AACCTGAGCTATAAGCAGGTT-3' (AY278951, 400-420, siRNA-400); and 5'-AACTTCTCTGCCAGTGATTTG-3' (AY278951, 793-813, siRNA-793). Double-stranded siRNAs were synthesized using the Silencer™ siRNA construction kit from Ambion Inc. (Austin, TX, USA). Briefly, the sense and antisense oligonucleotides targeting three different regions on the mouse Nanog with a 5'-CCTGTCTC-3' sequence complementary to the T7 promoter at the 3'-end of both oligonucleotides were synthesized. The sense and antisense siRNA transcripts were transcribed for 2 h in separate reactions with T7 RNA polymerase. The reactions were then mixed, and the combined reaction was incubated at 37℃ overnight. A single-strand specific ribonuclease and DNase digestion were used to eliminate the 5'- overhanging leader sequence and the DNA template, respectively. The resulting siRNAs were recovered from the mixture of nucleotides, enzymes, short oligomers, and salts in the reaction by column purification.

2. Cell Culture and Transfection

The P19 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco’s Modified Eagle’s Medium

(DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 100 units penicillin/mL and 100 μg streptomycin/mL. The cells were seeded in 12-well plates at a density of 2.5×10^4 cells/well. The cells were transiently transfected with the Nanog-siRNA duplex using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were transfected with 50 nM of the siRNA duplex using Lipofectamine 2000 at a ratio of 1:1 and incubated at 37℃ for 48 h. Gapdh negative (non-targeting) control siRNA which causes no effect on the mRNA and protein levels of Gapdh was purchased from Ambion Inc and used as a control for nonspecific effects such as nonsequence-specific siRNA effects, cytotoxicity of the transfection agent and/or the siRNA. The Gapdh negative control siRNA is a scrambled sequence that has no significant homology to the human, mouse, or rat genome.

3. RT-PCR and Real-Time PCR

Total RNAs were extracted from glyceraldehyde-3-phosphate dehydrogenase (Gapdh) negative control siRNA or Nanog siRNA-100 transfected P19 cells with the Trizol reagent (Invitrogen). Then, 0.5 μg of the total RNAs was treated with DNase (Promega, Madison, WI, USA) to remove the contaminated genomic DNA. The first-strand cDNA was synthesized from 0.5 μg of DNase-treated total RNA using 0.5 μg random hexamers, and 200 U of the Moloney murine leukemia virus reverse transcriptase (Invitrogen) at 37℃ for 60 min in a volume of 20 μL. The first strand cDNA (1 μL) was used for PCR amplification in a 25 μL reaction mixture. RT-PCR was performed under the following conditions: 1 cycle of 94℃ for 5 min; 30 cycles of 94℃ for 1 min, 60℃ for 40 sec, 72℃ for 40 sec and a final extension of 72℃ for 7 min. The PCR reaction was carried out for 27 cycles for Gapdh and for 30 cycles for other primers. After PCR, 15 μL aliquots of each PCR product was electrophoresed on a 1.5% agarose gel and the gel was analyzed using the Bio-Rad Gel Documentation System (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was performed using an iQ™ Cycler (Bio-Rad Laboratories); each reaction contained 25 μL of the iQ™ SYBER Green Supermix (Bio-Rad Laboratories), 3 μL of forward primer (5 μM), 3 μL of reverse primer (5 μM), 5 μL of a 1:20 dilution of a cDNA, and 14 μL H_2O. The PCR conditions included a denaturation step (95℃ for 3 min), amplification and quantification repeated 45 times (94℃ for 15 sec, 60℃ for 30 sec, and 72℃ for 30 sec), and melting curve analysis (55～95℃ with a heating rate of 0.05℃/sec). The primer sequences used for RT-PCR and real-time PCR are listed in Table 1. The measurement of gene expression was assayed in triplicate. The relative gene expression levels were quantified based on the Ct, and normalized to the reference gene Gapdh.

4. Statistical Analysis

The statistical analysis was performed using a Student's *t*-test and statistical significance was set *a priori* at $P<0.05$. All statistical values are expressed as the mean±standard deviation (SD). All statistical analyses were performed using SigmaStat3.1 software (version 3.1, SPSS Inc. Chicago, IL, USA).

RESULTS

1. Selection of Efficient Nanog siRNA Duplexes in P19 Embryonal Carcinoma Stem Cells

Previous studies have reported that downregulation of Nanog using the RNAi technique induced differentiation in human ES and EC cells (Hyslop et al., 2005). In this study, we generated three different siRNA duplexes using the siRNA Target Finder online tool (www.ambion.com) to inhibit the endogenous mouse Nanog expression in the P19 cells (Fig. 1). We next examined the silencing efficiency of siRNA duplexes targeted against the mouse Nanog gene by RT-PCR and quantitative real-time PCR. The Gapdh siRNA duplex (Ambion) was used as a negative control. The morphological change between the Nanog siRNA- and Gapdh siRNA-transfected P19 cells was not

Table 1. Primer sequences for RT-PCR and real-time PCR

Gene	Accession number	Primer sequences	Product size (bp)
Cripto	NM_011562	5'-TGGAT TGTTT GATTG ATGTT TACGG-3' 5'-TGTTT GGGTT TTCTG TGAGG-3'	203
Dab2	NM_23118	5'-TGAAGCAGACAGCCAGAAACA-3' 5'-CAACAGACAGGGATTTGATAGGG-3'	222
Fgf4	NM_010202	5'-GAGGCGTGGTGAGCATCTT-3' 5'-ACACTCGGTTCCCCTTCTTG-3'	213
Fgf5	NM_010203	5'-AAAGTCAATGGCTCCCACGAA-3' 5'-CTTCAGTCTGTACTTCACTGG-3'	465
Gapdh	NM_008084	5'-ACCACCATGGAGAAGGC-3' 5'-GGCATGGACTGTGGTCATGA-3'	234
Gata4	NM_008092	5'-CTGTCATCTCACTATGGGCA-3' 5'-CCAAGTCCGAGCAGGAATTT-3'	259
Gata6	NM_010258	5'-GAGCTGGTGCTACCAAGAGG-3' 5'-TGCAAAAGCCCATCTCTTCT-3'	193
Gcnf	AF390896	5'-CTGAACAACGAACCTGTCTC-3' 5'-TTGCTCTCTGAAGCCCTGTT-3'	400
Gp130	X62646	5'-ATTTGTGTGCTGAAGGAGGC-3' 5'-AAAGGACAGGATGTTGCAGG-3'	186
Isl1	NM_021459	5'-AGCAAGAACGACTTCGTGATG-3' 5'-GACTGAGAGGGTCTCCAGCTC-3'	187
Lif	NM_008501	5'-GCAACCTCATGAACCAGATC-3' 5'-CAACTTGGTCTTCTCTGTCC-3'	183
Nanog	AY278951	5'-CACCCACCCATGCTAGTCTT-3' 5'-ACCCTCAAACTCCTGGTCCT-3'	150
Oct3/4	NM_023633	5'-GGCGTTCTCTTTGGAAAGGTGTTC-3' 5'-CTCGAACCACATCCTTCTCT-3'	312
Rex1	NM_009556	5'-CAGGTTCTGGAAGCGAGTTC-3' 5'-GACAAGCATGTGCTTCCTCA-3'	255
Sox1	NM_009233	5'-CACAACTCGGAGATCAGCAA-3' 5'-TGTAATCCGGGTGTTCCTTC-3'	127
Sox2	NM_011443	5'-CACAGATGCAACCGATGCA-3' 5'-GGTGCCCTGCTGCGAGTA-3'	122
Stat3	NM_213659	5'-CAGAAAGTGTCCTACAAGGGCG-3' 5'-CGTTGTTAGACTCCTCCATGTTC-3'	340
Yes	X67677	5'-CCACAAAGGGAGCATCAGTT-3' 5'-GAATGGAATCTGCAGGCACT-3'	324
Zfp57	NM_009559	5'-TCACTTGTGCTGCCAAAGAC-3' 5'-TCCCGAGGAGTCCCTTACTT-3'	124

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1   ATGAGTGTGGGTCTTCCTGGTCCCCACAGTTTGCCTAGTTCTGAGGAAGCATCGAATTCT
61  GGGAACGCCTCATCAATGCCTGCAGTTTTTCATCCCGAGAACTATTCTTGCTTACAAGGG
121 TCTGCTACTGAGATGCTCTGCACAGAGGCTGCCTCTCCTCGCCCTTCCTCTGAAGACCTG
181 CCTCTTCAAGGCAGCCCTGATTCTTCTACCAGTCCCAAACAAAAGCTCTCAAGTCCTGAG
241 GCTGACAAGGGCCCTGAGGAGGAGGAGAACAAGGTCCTTGCCAGGAAGCAGAAGATGCGG
301 ACTGTGTTCTCTCAGGCCCAGCTGTGTGCACTCAAGGACAGGTTTCAGAAGCAGAAGTAC
361 CTCAGCCTCCAGCAGATGCAAGAACTCTCCTCCATTCTGAACCTGAGCTATAAGCAGGTT
421 AAGACCTGGTTTCAAAACCAAAGGATGAAGTGCAAGCGGTGGCAGAAAAACCAGTGGTTG
481 AAGACTAGCAATGGTCTGATTCAGAAGGGCTCAGCACCAGTGGAGTATCCCAGCATCCAT
541 TGCAGCTATCCCCAGGGCTATCTGGTGAACGCATCTGGAAGCCTTTCCATGTGGGGCAGC
601 CAGACTTGGACCAACCCAACTTGGAGCAGCCAGACCTGGACCAACCCAACTTGGAACAAC
661 CAGACCTGGACCAACCCAACTTGGAGCAGCCAGGCCTGGACCGCTCAGTCCTGGAACGGC
721 CAGCCTTGGAATGCTGCTCCGCTCCATAACTTCGGGGAGGACTTTCTGCAGCCTTACGTA
781 CAGTTGCAGCAAAACTTCTCTGCCAGTGATTTGGAGGTGAATTTGGAAGCCACTAGGGAA
841 AGCCATGCGCATTTTAGCACCCCACAAGCCTTGGAATTATTCCTGAACTACTCTGTGACT
901 CCACCAGGTGA
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Fig. 1. The nucleotide sequence of mouse Nanog. The numbers on the left indicate the nucleotide base pair position. The underlined nucleotide sequences indicate siRNA target regions selected using the siRNA Target Finder online tool (Ambion). The start codon and stop codon sequences are indicated in bold letters.

distinct at 48 h after transfection (data not shown). However, the RT-PCR analysis showed that three kinds of Nanog siRNAs, siRNA-100, siRNA-400 and siRNA-793 remarkably inhibited the endogenous Nanog expression compared to the Gapdh negative control siRNA (Fig. 2A). Quantitative real-time PCR analysis further confirmed the significant downregulation of the Nanog gene; there was a 30.68±1.21%, 67.86±11.31% and 52.97±11.51% reduction of Nanog in the cells transfected with Nanog siRNA-100, siRNA-400 and siRNA-793, respectively, compared with cells treated with Gapdh as a negative control siRNA (Fig. 2B). Nanog siRNA-100 was the most effective for inhibiting endogenous Nanog expression (Fig. 2). Therefore, the Nanog siRNA-100 duplex was finally selected to investigate the effects on the knockdown of endogenous Nanog expression. Collectively, these results showed that the Nanog siRNA duplexes, generated in this study, were effective for the suppression of endogenous Nanog expression.

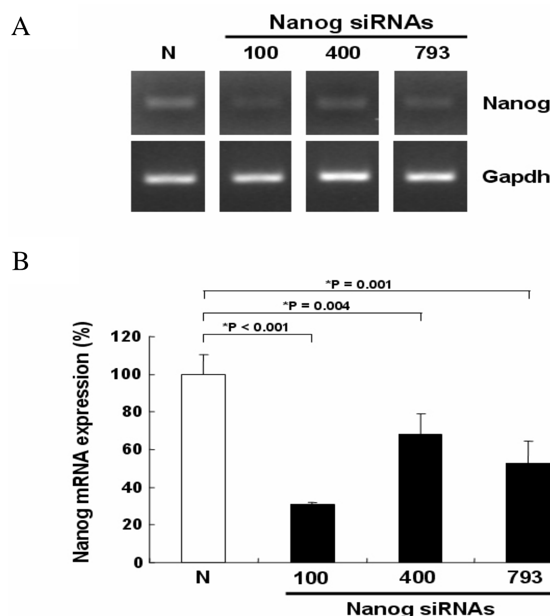

Fig. 2. Downregulation of Nanog expression in P19 cells by RNA interference. P19 cells were cultured in 12-well plates at a density of 2.5×10^4 cells/well and transfected with 50 nM of the Nanog siRNA duplexes (siRNA-100, siRNA-400 and siRNA-793) or a Gapdh negative control siRNA using Lipofectamine 2000. After a 48 h transfection, Nanog expression was assessed by RT-PCR (A) and real-time PCR (B). The average value of Nanog mRNA was normalized to that of Gapdh for each sample. The data represent the mean SD from three experiments. Statistical significance of the results was assessed using the Student's *t*-test (**P*<0.05). N, Gapdh negative control siRNA siRNA, small interfering RNA.

2. Nanog Knockdown in P19 cells Reduces the Expression of Pluripotent Stem Cell Markers

Loss of pluripotency and extraembryonic endoderm differentiation in Nanog null cells were observed (Mitsui et al., 2003). We investigated the mRNA expression levels of various cell lineage markers by RT-PCR or real-time PCR to identify the putative downstream target genes for Nanog after siRNA-mediated knockdown of the Nanog gene. We transfected P19 cells for 48 h with either Nanog siRNA-100 or the Gapdh negative control siRNA and conducted RT-PCR analysis for markers of pluripotency such as Cripto, fibroblast growth factor (Fgf4), Oct3/4, Rex1, Sox1,

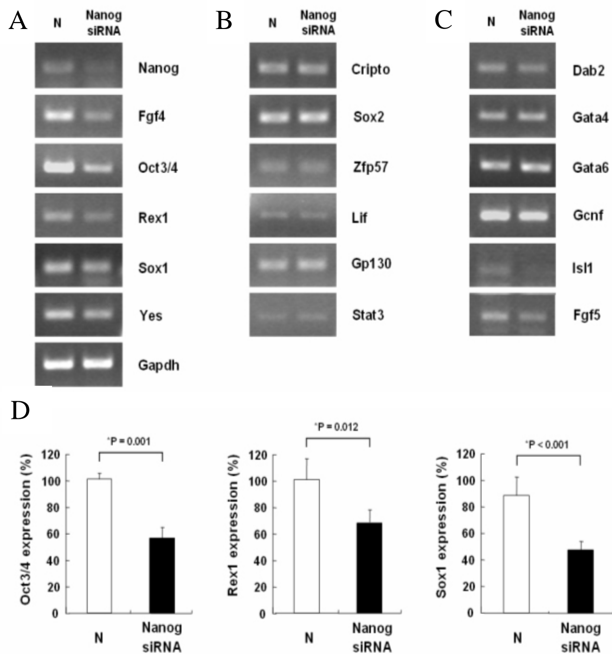

Fig. 3. Nanog knockdown in P19 cells results in reduction of pluripotency markers. P19 cells were cultured in 12-well plates at a density of 2.5×10^4 cells/well and transfected either with 50 nM of Gapdh negative control siRNA or Nanog siRNA-100. At 48 h after transfection, the expression of cell lineage markers was assessed by RT-PCR (A, B and C) or real-time PCR (D). (A) Nanog downregulation resulted in reduction of some pluripotency markers (Fgf4, Oct3/4, Rex1, Sox1, Yes). (B) Expression of some pluripotency markers and Lif/Stat3 pathway molecules (Cripto, Gcnf, Sox2, Zfp57, Lif, Gp130, Stat3) was not affected by Nanog knockdown. (C) Nanog downregulation resulted in the reduction of the ectoderm markers, Fgf5 and Isl1. (D) Nanog downregulation resulted in a significant reduction in some pluripotency markers (Oct3/4, Rex1 and Sox1). The average value of mRNA expression of pluripotency markers was normalized to that of the Gapdh for each sample. The data represent the mean SD from three experiments. Statistical significance of the results was assessed using the Student's *t*-test ($^*P<0.05$). Dab2, disabled-2; Fgf4, fibroblast growth factor 4; Gcnf, germ cell nuclear factor; Gp130, glycoprotein 130; Isl1, islet-1; Lif, leukemia inhibitory factor; N, Gapdh negative control siRNA Oct3/4, octamer-binding transcription factor 3/4; STAT3, signal transducer and activator of transcription-3; siRNA, small interfering RNA; Sox1, Sry (sex determining region Y)-box 1; Zfp57, zinc finger protein 57; Yes, yamaguchi sarcoma viral oncogene homolog.

Sox2, yamaguchi sarcoma viral oncogene homolog (Yes) and the zinc finger protein 57 (Zfp57), of the leukemia inhibitory factor/signal transducer and activator of transcription-3 (Lif/Stat3) signaling and of cell lineage differentiation such as Afp, Coup-tf1, Dab2, Fgf5, Gata2, Gata4, Gata6, germ cell nuclear factor (Gcnf), Hnf1-β, Hnf4-α, Ihh, Isl1, LamininB1, Tm and Ttr. We found that the expression of pluripotency markers such as Fgf4, Oct3/4, Rex1, Sox1 and Yes was downregulated at 48 h after transfection with Nanog siRNA-100 compared to the cells transfected with the Gapdh negative control siRNA (Fig. 3A). However, the expression of other pluripotency markers such as Cripto, Sox2 and Zfp57 was not affected by Nanog knockdown (Fig. 3B). In addition, the RT-PCR analysis of the Lif/Stat3 pathway molecules showed no differences between the Nanog siRNA-100 and the Gapdh negative control siRNA cells at 48 h after transfection (Fig. 3B). These results suggested that Nanog downregulation did not affect these molecules. Nanog knockdown in P19 cells did not induce the expression of cell lineage markers Dab2, Gata4, Gata6 and Gcnf (Fig. 3C). By contrast, expression of the primitive ectoderm marker Fgf5 and the neuroectodermal marker Isl1 were reduced by the Nanog knockdown (Fig. 3C). Expression of parietal endoderm markers LamininB1 and Tm, and endoderm transcription factors Coup-tf1, Hnf1-β, Hnf4-α and Gata2, and of visceral endoderm markers Afp, Ihh and Ttr was not detectable or only very weak in P19 cells (data not shown).

To quantify the mRNA expression of the pluripotency markers after Nanog knockdown, we performed real-time PCR analysis on the markers of pluripotency. The stem cell markers such as Oct3/4 (1±0.04% vs. 0.56±0.08%), Rex1 (1±0.16% vs. 0.68±0.10%) and Sox1 (1±0.17% vs. 0.54± 0.07%) in P19 cells transfected with Nanog siRNA-100 were significantly downregulated compared to the cells transfected with the Gapdh negative control siRNA (Fig. 3D).

DISCUSSION

The transcription factor, Nanog is a major regulator of self-renewal and of the pluripotent state in ES cells as well as EC cells. In this study, we used P19 embryonal carcinoma stem cells to elucidate the regulatory mechanisms of Nanog and identify the potential downstream targets of Nanog. It has been suggested that siRNA target sequences should not be positioned in the 5' untranslated region or near the start codon (Elbashir et al., 2002). In addition, confirmation of effective siRNA molecules should be validated experimentally with several siRNAs across different regions of a specific mRNA. Thus, we designed three siRNA duplexes from mouse Nanog sequences using the siRNA Target Finder online tool (www.ambion.com). We found that Nanog siRNA-100, among three siRNAs used in this study, was the most effective in inhibiting of endogenous Nanog expression (about 70% inhibition). Persengiev et al. (2004) reported that the nonspecific effects by siRNA occurred in a concentration dependent fashion. Significantly, 100 nM siRNA was found to be a concentration at which nonspecific effects occur (Semizarov et al., 2003; Persengiev et al., 2004). Therefore, we used 50 nM of siRNA duplexes to avoid the potential nonspecific effects of the siRNA at higher concentrations.

In this study, transient reduction of Nanog expression by siRNAs led to downregulation of the markers for undifferentiated ES and EC cells such as Oct3/4, Rex1, Sox1, Fgf4 and Yes. However, the expression of Sox2 was not affected. Consistent with this observation, Hyslop et al. (2005) reported that Nanog downregulation induced Oct3/4 downregulation on day 4 after transfection in human ES cells; however, no dewnregulation of Sox2 was observed in this study. Moreover, recent studies have shown that Sox2 binds to the Oct3/4 gene equally well in both F9 and P19 EC cells, but fails to activate the Oct3/4 promoter in the P19 EC cells (Mallanna et al., 2008). These findings suggest a limited ability of Sox2 to stimulate the Oct3/4 promoter in the P19 cells. By contrast, Oct3/4, Sox2 and Nanog were thought to collaborate to form a transcriptional network for the regulation of pluripotency in mouse and human ES cells (Boyer et al., 2005). Indeed, Liang et al. (2008) demonstrated that Nanog and Oct3/4 interact with each other and associate with proteins from multiple repression complexes in mouse ES cells. Taken together, the discrepancies in the results can be attributed to differences in the mechanisms underlying Sox2 regulation between ES cells and EC cells. Interestingly, we found that Sox1 expression was reduced after the Nanog siRNA-transfected P19 cells. Sox1, one member of the SoxB1 subfamily (Sox1, Sox2 and Sox3) is a known marker of embryonic neural stem cells (Aubert et al., 2003). Previous studies (Pevny et al., 1998) have reported that the onset of Sox1 expression is itself sufficient to impart a neural fate on the competent ectodermal P19 cells. In this context, our observations suggest that Nanog may suppress Sox1 expression to prevent the differentiation of P19 cells into a neural lineage.

Rex1 is a marker for the pluripotent state in ES cells and EC cells (Hosler et al., 1989; Rogers et al., 1991) and identified as a direct target of Nanog that is augmented by Sox2 and Oct3/4 in the P19 cells (Shi et al., 2006). In accordance with previous reports, we noted that Rex1 expression resulted in reduction in the Nanog siRNA-transfected P19 cells. Similarly, Rex1 expression was reduced in Nanog-downregulated human EC cells but not in human ES cells, suggesting that Rex1 may interact differently with Nanog in ES cells than in EC cells (Hyslop et al., 2005). We also found that Yes, a member of the Src family of non-receptor tyrosine kinases, which was enriched in ES cells (Ramalho-Santos et al., 2002) was reduced after the Nanog siRNA-transfected P19 cells. Our observation is consistent with a previous report that showed that Nanog siRNA induced a reduction in the mRNA levels of Yes in mouse ES cells, neural stem cells and hematopoietic stem cells (Ramalho-Santos et al., 2002). Moreover, Yes siRNA reduced the expression of Nanog mRNA and increased the expression of Gcnf in mouse ES

cells (Anneren et al., 2004). Therefore, our findings and previous reports suggest that Nanog may interact with Yes for the maintenance of mouse P19 EC and ES cells in the undifferentiated state. On the other hand, expression of the markers involved in the Lif/Stat3 pathway was not affected by the knockdown of Nanog in P19 cells. This finding is consistent with previous reports that showed that Nanog maintained the pluripotency of mouse ES cells independently of Lif/Stat3 (Mitsui et al., 2003). Cripto is known as a key regulator of ES cell fate. The different timing of Nodal-dependent Cripto signaling induces a different cell fate (Minchitti, 2005). Liu et al. (2005) demonstrated that Cripto can regulate the critical stem cell gene Nanog in P19Cl6 stable cell expressing siRNA directed at the Cripto transcripts, suggesting that Cripto may be a gene upstream to the Nanog.

Nanog was predicted to function as a repressor of Gata6; the Nanog binding motif was identified in the Gata6 promoter in both mouse and human ES cells (Mitsui et al., 2003; Boyer et al., 2005). Indeed, Terada and colleagues (Singh et al., 2007) demonstrated that Nanog directly repressed Gata6 expression through its binding to the promoter region of the Gata6 gene. However, we did not observe the induction of Gata6 at 48 h after transfection with Nanog siRNA in the P19 cells under our experimental conditions. Furthermore, the expression of the endodermal markers was so weak or not detectable in the Nanog-downregulated P19 cells. Expression of Gcnf, a gene that represses Oct3/4 gene activity in differentiating cells (Fuhrmann et al., 2001) was also not affected by Nanog inhibition. These results show no distinct change in the Nanog-downregulated P19 cells. Taken together, these results suggest that upregulation of cell lineage markers may be detected after 48 h of transfection with Nanog siRNA under our experimental condition. Hyslop et al. (2005) reported that endodermal transcription factors such as Gata2, Gata4 and Gata6 were upregulated on day 4 after transfection in human ES and EC cells after transfection with Nanog siRNA. In addition Nanog^{high} cells expressed the extraembryonic endodermal markers Gata6 and Sparc after 4 days of differentiation in mouse ES cells (Singh et al., 2007). By contrast, Gata6 expression was upregulated as early as 24 hours, whereas upregulation of Gata4 was not detected until 48 hours (Hough et al., 2006). The differences in these results might be attributed to various factors, including differences in kinetics, the cell lines, or culture conditions including the cell densities. Another possible explanation might be attributed to a gene dosage effect. Indeed, levels of Nanog inhibition in mouse ES cells could affect the cell fate, suggesting that Nanog may function in a dose-dependent manner to influence the cell fate (Hough et al., 2006). Taken together, our results are overall consistent with previous reports that Nanog maintains the pluripotent state in both ES and EC cells (Chambers et al., 2003; Mitsui et al., 2003; Hyslop et al., 2005; Chen et al., 2006) although some differences in regulation of pluripotency and differentiation markers following the knockdown of Nanog were found.

In conclusion, we demonstrated that the transient inhibition of Nanog expression, by RNAi in P19 cells, was sufficient to modulate the expression of the pluripotent markers involved in the self-renewal of EC stem cells. These results provide valuable clues for identifying the potential downstream targets of Nanog as well as for understanding the function of Nanog in P19 EC stem cells.

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