Nanog expression is negatively regulated by protein kinase C activities in human cancer cell lines

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Nanog is a transcription factor that is essential for the maintenance of pluripotency of the embryonic stem cells. Nanog has been shown to be expressed in various kinds of human tumors, suggesting a role in tumorigenesis. In this study, we found that Nanog expression was upregulated by inhibition of protein kinase C (PKC) activity in six human cancer cell lines examined. In a Nanog non-expressing human nasopharyngeal carcinoma cell line, NPC-076, Nanog mRNA level and protein level were both induced and dose-dependently promoted by exposure to PKC inhibitors. Knockdown experiments showed that PKCa and PKCb were two subtypes exerted most of the effect. The reporter assay showed that Nanog promoter activity was promoted by exposure of the cells to PKC inhibitors and the effect was dependent on the presence of the Octamer–Sox composite element. The involvement of Octamer–Sox composite element was further supported by the observation that silencing of Oct4 and Sox2 in NPC-076 cells attenuated the effects of PKC inhibitors. In Nanog-expressing human embryonal carcinoma cell lines, NT2/D1 and NCCIT, Nanog expression was suppressed by exposure to PKC activator Phorbol-12-myristate-13-acetate (PMA). Further study showed that overexpression of PKCa elicited a repressive effect on Nanog expression in NT2/D1 cells. Consistently, mutation of the Octamer–Sox composite element abolished the suppressive effect by PKC activator. Nanog expression was of cellular significance in that ectopic expression in NPC-076 stimulated cell proliferation and knockdown of the endogenous Nanog expression in NT2/D1-suppressed cell proliferation.

Introduction

Several transcription factors have been shown to play roles in regulating pluripotent and self-renewing characteristics of the embryonic stem (ES) cells. These transcription factors include, but not limit to Nanog, Sox2 and Oct4 (1). Through cooperative interactions, Nanog, Sox2 and Oct4 have been shown to drive pluripotent-specific expression of downstream target genes (1,2). Nanog is a homeobox-containing transcription factor that is essential for maintaining the pluripotency of the inner cell mass (3), and is specifically expressed in human ES cells (4), primordial germ cells (5) and fetal testis (6). The transcription of Nanog gene is regulated by Oct4-Sox2 complex, through an adjacent pair of highly conserved Oct4- and Sox2-binding elements in the 5′-flanking region of the Nanog promoter (7). Downregulation of Nanog expression was reported to induce ES cells differentiation, whereas Nanog overexpression inhibited differentiation (4). Interestingly, overexpression of Nanog was shown to promote proliferation and transformation of NIH3T3 cells (8). Moreover, Nanog and its pseudogene, NanogP8, were expressed not only in human germ cell tumors (9) but also in a number of human cancers, including colorectal cancer (10), seminomas and breast carcinomas (11), oral cavity carcinomas (12), ovary carcinomas (13), renal carcinomas (14) and in malignant cervical epithelial cells (15). Although overexpression of Nanog has been shown to be strongly correlated with poor prognosis, and lymph node metastasis in colorectal cancers (10), nevertheless, its underlying has been largely unclear.

Protein kinase C (PKC) comprises a family of serine/threonine kinases, at least 12 isoforms have been reported that are involved in cellular signaling pathways regulating important cell functions, including cell proliferation and differentiation (16). PKC family members have been divided into three subclasses: (i) The classical PKCs (α, β, γ and δ) that bind phorbol esters and are Ca2+ dependent for activity; (ii) the novel PKCs (ε, η, ι and θ) that bind phorbol esters but do not depend on Ca2+ for activity; (iii) the atypical PKCs (ζ, ξ, ζ and μ) that neither bind phorbol esters nor Ca2+ dependent (17). The phorbol ester PMA, which activates the classical and novel PKCs, has been shown to induce macrophage differentiation of the myelomonocytic cell line U937 (18) and the promyelocytic cell line HL60 (19). Activation of PKC by PMA has also been shown to promote megakaryocytic differentiation and inhibits erythroid differentiation (20,21). Of the isoforms, PKCα and PKCβ have been suggested to play role in normal epidermal differentiation. PKCα inhibits, whereas PKCβ promotes cell differentiation. PKC activity was increased in the human embryonal carcinoma cell line NT2/D1 cells when induced to undergo differentiation by retinoic acid (22). A dramatic induction of PKCα was observed after neuronal differentiation. Furthermore, previous report showed that PKC-ε inhibitors sustain self-renewal of mESCs under hypoxia, suggesting that inhibition of PKC-ε activity blocks the early differentiation of mESCs (23). Recently, Gao et al. (24) reported that the protein levels of PKCα, PKCβ and PKCε were upregulated in ES cells on days 3 and 5 after retinoic acid exposure. Taken together, these studies suggest that the three PKC isoforms are necessary for the initiation and maintenance of the differentiated state in these cells. Nanog has been reported to play role in the maintenance of the pluripotent ES cell phenotype and in cancer progression (25). However, not much has been known on how Nanog gene expression is regulated. In this study, we provide experimental results to show that PKC activities were involved in the regulation of Nanog expression. We examined the Nanog expressions in six human cancer cell lines including NPC-076, HepG2, HT1376, SW620, T24 and Hep3B, on inhibition of PKC activity. We found that Nanog gene expression was increased by treatment with PKC inhibitor in all six human cancer cell lines. We also showed that treatment of PKC inhibitors in a Nanog non-expressing human cancer cell line NPC-076, dramatically induced Nanog expression at both mRNA and protein levels. The involvement of PKC activity in the regulation of Nanog expression was further confirmed by knockdown of PKCα or PKCβ with small interfering RNA (siRNA) in NPC-076 cells. In contrast, in the Nanog constitutive human embryonal carcinoma cells (NT2/D1 and NCCIT), the Nanog expression was suppressed by treatment with PKC activator. Expression of ectopic PKCα in NT2/D1 cells repressed the expression of Nanog and TRA-1–60, a human pluripotent stem cell marker. Further study showed that the Octamer–Sox composite element in the Nanog promoter is involved in the suppression of Nanog expression by PKC.

Our findings indicated that PKC plays important roles in the regulation of Nanog expression. Because the expression of Nanog appeared to play regulatory role in tumor cell proliferation, we suggested a possible implication of PKC–Nanog pathway in tumor progression.

Abbreviations: ANOVA, analysis of variance; AP-1, activator protein-1; BMP, bone morphogenetic protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LIF, leukemia inhibitory factor; NPC, nasopharyngeal carcinoma; PKC, protein kinase C.
Materials and methods

Cell line and cell culture

Human nasopharyngeal carcinoma (NPC) cell line NPC-076 was established from poorly differentiated NPC biopsy (26). Human liver hepatocellular carcinoma cells (HepG2 and Hep3B), human bladder carcinoma cells (HT1376 and T24), human cervical carcinoma cells (SW-620) and human embryonal carcinoma cells (NT2/D1 and NCCIT) were obtained from Food Industry Research and Development Institute, Taiwan. NPC-076, HepG2, Hep3B, HT1376, T24 and SW-620 cells were maintained in basal medium (Dulbecco’s modified Eagle’s medium/F-12 at 3:1, vol/vol; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 5% CO₂ under 5% CO₂ at 37°C. Human liver hepatocellular carcinoma cells (HepG2 and Hep3B), human bladder carcinoma cells (HT1376 and T24), human colorectal carcinoma cells (SW620) and human embryonal carcinoma cells (HepG2 and Hep3B), human bladder carcinoma cells (HT1376) and human embryonal carcinoma cells (HepG2 and Hep3B) were incubated overnight with PKC inhibitors chelerythrine chloride or staurosporine at the indicated concentrations. Twenty-four hours after incubation, the total RNA was harvested and used for western blot analysis or RT–PCR using primers as described in the text. GAPDH gene was used as an internal control for RNA quality and loading. For western blot analysis, NPC-076 cells were incubated with chelerythrine chloride (Fig. 1B) or staurosporine (Figure 1C) at the indicated concentrations. Twenty-four hours after incubation, the total RNA was harvested and used for western blot analysis or RT–PCR using primers as described in the text. GAPDH gene was used as an internal control for RNA quality and loading. For western blot analysis, NPC-076 cells were incubated with chelerythrine chloride (Fig. 1B) or staurosporine (Figure 1C) at the indicated concentrations. The immunoreactive bands were analyzed with a densitometer, and the ratio of the Nanog to GAPDH signal was calculated. The Nanog to GAPDH ratio in the absence of treatment was set as 1. Data are mean ± SD of three independent experiments. Significance was tested using one-way analysis of variance (ANOVA) with Holm–Sidak method post hoc test, where *P < 0.05 and **P < 0.01.

Semi-quantitative RT–PCR

Total RNA from cells was isolated in TRI Reagent (Molecular Research Center, Cincinnati, OH), and a two-step RT–PCR method was used to measure the expression levels of the desired genes. Oligo-(dT)₃ was incubated at room temp for 30 min to measure the expression levels of the desired genes. Oligo-(dT)₃ was incubated at room temp for 30 min and 1 μl of DNase I Inactivation Reagent was then added. The mixture was further incubated for 2 min at room temp and then heated at 65°C for 2 min to break down any secondary structures. The reaction mixture was cooled rapidly on ice and 4 μl of 5× RT Buffer, containing 10 mM dithiothreitol, 100 μM deoxynucleoside triphosphates and 200 U Superscript III Reverse Transcriptase (Invitrogen) were then added. The reaction mixture was further incubated at 50°C for 1 h and the reaction was stopped by heating at 70°C for 15 min. The PCR was performed for 25–35 cycles at 95°C (1 min), 60°C (1 min) and 72°C (1 min) in 2× GoTaq® Green Master Mix (Promega, Madison, WI). The primers used for Nanog were as follows: sense, 5′-GGATCCAGCTGTCCTCCAAAGCTTG-3′; antisense, 5′-CTGGAGGC TGGGGTTTCTGTCTC-3′. The primers used for miR302b-367 cluster were as follows: sense, 5′-GGGCTCCTTTCGAACTTAAAC-3′; antisense, 5′-ATTTGTTCATATTGTGATACTACCC-3′ (27). Two internal control genes were also amplified; the β-actin gene primer pairs used were as follows: sense, 5′-GGCAAGAGATGACCCGATCTAGTT-3′; antisense, 5′-GCTTCTTCATTAATGTCAGCGAT-3′, and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene primer pairs used were as follows: sense, 5′-GAAAAGTGAAGTCGGAGGT-3′; antisense, 5′-GAAGATGTTGATGGGATTTC-3′. The PCR products were resolved by 1.5% agarose gel and stained by ethidium bromide.

Construction of Nanog promoter–luciferase constructs and expressing vectors

A human Nanog promoter fragment from −993 to +231 relative to the transcriptional start site was subcloned by PCR amplification. The PCR reaction was carried out with the sequence specific primer pair, the primers were designed to contain a XhoI site and a HindIII site for the subsequent cloning reactions, they were as follows: forward, 5′-GGCAAGAGATGACCCGATCTAGTT-3′; antisense, 5′-GCTTCTTCATTAATGTCAGCGAT-3′, and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene primer pairs used were as follows: sense, 5′-GAAAAGTGAAGTCGGAGGT-3′; antisense, 5′-GAAGATGTTGATGGGATTTC-3′. Two inter

A. Chelerythrine Chloride (μM)

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Fig. 1. Nanog is induced by PKC inhibitor in human cancer cell lines. (A) Human cancer cell lines (NPC-076, HepG2, HT1376, SW620, T24 and Hep3B) were incubated overnight with PKC inhibitors chelerythrine chloride. Cell lysates were prepared 24 h after treatment and were subjected to western blot analysis with anti-Nanog and anti-GAPDH antibodies. (B and C) NPC-076 cells were incubated with chelerythrine chloride (Figure 1B) or staurosporine (Figure 1C) at the indicated concentrations. Twenty-four hours after incubation, the total RNA was harvested and the RT–PCR assay was performed. PCR fragments were amplified by RT–PCR using primers as described in the text. GAPDH gene was used as an internal control for RNA quality and loading. For western blot analysis, NPC-076 cells were incubated with chelerythrine chloride (Figure 1B) or staurosporine (Figure 1C) at the indicated concentrations. The immunoreactive bands were analyzed with a densitometer, and the ratio of the Nanog to GAPDH signal was calculated. The Nanog to GAPDH ratio in the absence of treatment was set as 1. Data are mean ± SD of three independent experiments. Significance was tested using one-way analysis of variance (ANOVA) with Holm–Sidak method post hoc test, where *P < 0.05 and **P < 0.01.
Regulation of Nanog expression by protein kinase Cs

was mutated from CTTTTGCAATTCAATG to CTTTCAATTCAATG. The pGL3-Nanog-mut-Sox2-Luc vector containing a substitution mutation in the SOX2-binding site was mutated from CTTTTGCAATTCAATG to CTTTCAATTCAATG. The synthetic oligonucleotide primers used were as follows: sense, 5’-ACCTGTGACTTCTTCAATCCAATGGCCCTG-3’ and antisense, 5’-GAAGGCTATGGGAATGCTGAGT-3’ for pGL3-Nanog-mut-Oct4-Luc; sense, 5’-AGCTTCTTTGTATTAAACCTGGGCTTGTAGAC-3’ and antisense, 5’-GTCTACCAAGGCGAGTTAATGCAAAGTAGAGT-3’ for pGL3-Nanog-mut-Oct2-Luc. PCRs were performed at three temperature cycles of 95°C (30 s), 55°C (60 s) and 68°C (8 min) for a total of 12 cycles. After PCR, the methylated parental DNA templates were digested with 20 U DpnI (NEB, Schwalbach, Germany) at 37°C overnight. The DNA fragment containing the desired mutation was transformed into competent Escherichia coli JM109 cells, which are capable of repairing the nicked DNA. The presence of the desired mutation was verified by direct DNA sequencing.

**Transient transfection and luciferase assay**

Transient transfection of luciferase reporter plasmids was performed using Lipofectamine 2000 (Invitrogen) according to the protocol recommended by the supplier. Cells were seeded in 12-well tissue culture plates at 2 × 10^5 L/ml and transfected with reporter plasmids containing firefly luciferase by lipofection. 24 h after transfection, the cells were exposed to DNA-lipofectamine 2000 mixtures of transfection, the cells were exposed to DNA-Lipofectamine 2000 mixtures containing 0.5 μg of Nanog–luciferase reporter plasmid and 0.5 μg of pSV-β-galactosidase control vector (Promega, Madison, WI). To inhibit the PKC activity, the NPC-076 cells were incubated with the desired PKC inhibitors in growth medium containing 5% bovine serum. Cells were incubated for the indicated hours, rinsed with phosphate-buffered saline and lysed in 150 μl 1× reporter lysis buffer (Promega). Lysates were used directly for the Luciferase activity assay (Promega), performed according to the manufacturer’s protocols.

**Preparation of cell lysate and western blot analysis**

To prepare the total cell lysate, the cells were washed with ice-cold phosphate-buffered saline and lysed in Mammalian Protein Extraction Reagent (M-PER; Pierce, Rockford, IL). Lysis buffer contained 10 mM NaF, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and a cocktail of protease inhibitors (Sigma, Milwaukee, WI). Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Protein samples were fractionated on a 10% sodium dodecyl sulfate–polyacrylamide gel and blotted onto Immobilon(TM)-P membranes (Millipore, Bedford, MA). The membranes were blocked in TBB8 and reacted with primary antibodies (1:1000, respectively, for Nanog, Oct4, Sox2, PKCα, PKCδ, PKCε, TRA-1–60 and 1:30 000 for GAPDH) overnight at 4°C. The membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000 in Tween-20Tris buffered saline (TTBS)), and the immunoreactive protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany). Primary antibodies used including Nanog polyclonal antibody (Abcam, Cambridge, UK), GAPDH monoclonal antibody, Oct4 monoclonal antibody, Sox2 polyclonal antibody, TRA-1–60 monoclonal antibody (Chemicon, Temecula, CA) and PKC isoforms (α, δ and ε) antibodies (PKC sampler kit; BD Transduction Laboratories, San Jose, CA).

**Small interfering RNA transfection**

The sense siRNA sequences were purchased from MWG-Biotech (AG, Germany), including: PKCα siRNA, 5’-GCACAAUGUUCACUAUCAC-3’, PKCδ siRNA, 5’-GAUGAAGGAGGCUCUCAGC-3’, PKCε siRNA, 5’-GAUGAAGGAGGCUCUCAGC-3’, PKCε siRNA, 5’-GCACAAUGUUCACUAUCAC-3’, PKCδ siRNA, 5’-GAUGAAGGAGGCUCUCAGC-3’. PKC

**Fig. 2.** Nanog expression is dramatically increased by knockdown of PKCα or PKCδ in NPC-076 cells. (A) The NPC-076 cells were plated in 60 mm dish till 60% confluence and were transfected with siRNA (50 nM) against PKCα, PKCδ and PKCε or with non-silencing control siRNA (siNon). Cell lysates were prepared 24 h after treatment and were subjected to western blot analysis with anti-Nanog, anti-PKCα, anti-PKCδ, anti-PKCε and anti-GAPDH antibodies. To compare the relative band intensities, Nanog signals were normalized against GAPDH by densitometry. The ratio in the non-silencing control siRNA-transfected cells was set as 1. (B) NPC-076 and HepG2 cells were treated with or without different concentrations of rottlerin (5 and 15 μM). Cell lysates were prepared 24 h after treatment and were subjected to western blot analysis with anti-Nanog and anti-GAPDH antibodies. Band intensity is quantitated by densitometry using NIH ImageJ. Data are mean ± SD of three independent experiments. Significance was tested using one-way ANOVA with Holm–Sidak method post hoc test, where *P < 0.05 and **P < 0.01.
Regulation of Nanog expression by protein kinase Cs

siRNA, 5'-GCCCCUAAAGACAAUGAAG-3', Nanog siRNA, 5'-CCAGAC CUGGAAACAAUUAAC-3', Oct4 siRNA, 5'-CAUGUGUAAGCUCCGG CCC-3', Sox2 siRNA, 5'-aacaagacUcaUgaag-3' and control non-silencing siRNA, 5'-UUCUCCGAACGUGACGU-3'. The cells were grown in OPTI-MEM till 60% confluence and cotransfected with desired siRNA (50 nM) using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, the cells were harvested and cell lysates were prepared and used for western blot assay.

Fig. 3. Octamer–Sox composite element is involved in the PKC inhibitor-induced transactivation of Nanog gene transcription in NPC-076 cells. (A) The NPC-076 cells were transfected with 500 ng of pGL3-Nanog-Luc in the presence of increasing concentrations of chelerythrine chloride. The cells were cotransfected with pSVβ-galactosidase vector, and the β-galactosidase activity was used to normalize the luciferase activity. Luciferase activity is presented as fold induction relative to chelerythrine chloride-untreated cells. Data are means ± SD from triplicate analysis. Significance is tested using one-way ANOVA with Holm–Sidak method post hoc test, where ** P < 0.01 versus Not added (NA). (B) The expression of Oct4 and Sox2 are enhanced by PKC inhibitors in NPC-076 cells. NPC-076 cells were incubated with chelerythrine chloride at the indicated concentrations. Cell lysates were prepared 24 h after treatment and were subjected to western blot analysis with anti-Nanog, anti-Oct4, anti-Sox2 and anti-GAPDH antibodies. Figures are representative of at least three independent experiments. (C) The NPC-076 cells were cotransfected with pGL3-Nanog-Luc (0.5 μg/ml), and pCMV-Oct4 or pCMV-Sox2 (both at 0.2 μg/ml) or together with pCMV-Oct4 and pCMV- Sox2 for 24 h. The pCMV-Empty vector was added when necessary to keep the total plasmid concentration constant. The cells were also cotransfected with a pSVβ-galactosidase vector, and the β-galactosidase activity was used to normalize the luciferase activity. Luciferase activity is presented as the fold induction relative to pCMV-Empty-transfected cells. Data are means ± SD from triplicate analysis. * P < 0.05 (t-test) compared with the control. (D) The cells were transfected with either wild-type pGL3-Nanog-Luc, pGL3-Nanog-mut-Oct4-Luc or pGL3-Nanog-mut-Sox2-Luc for 24 h. The three base substitutions in the pGL3-Nanog-mut-Oct4-Luc and pGL3-Nanog-mut-Sox2-Luc are underlined. The cells were also cotransfected with pSVβ-galactosidase vector. The cells were treated with increasing concentrations of chelerythrine chloride for 24 h after transfection. Luciferase activity is presented as the fold induction relative to chelerythrine chloride-untreated cells. Data are means ± SD from triplicate analysis. Asterisks indicate a significant difference compared with the wild-type control. * P < 0.05 and ** P < 0.01 (t-test). (E) The embryonal carcinoma cell line NT2/D1 was transfected with either pGL3-Basic, pGL3-Nanog-Luc, pGL3-Nanog-mut-Oct4-Luc or pGL3-Nanog-mut-Sox2-Luc. The cells were also cotransfected with a pSVβ-galactosidase vector, and the β-galactosidase activity was used to normalize the luciferase activity. Luciferase activity is presented as fold induction relative to pGL3-Basic-transfected cells. Data are mean ± SD from three independent analyses.
3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay

The NPC-076 cells were cultured to 70% confluence in 60 mm culture plates and were transfected with pCMV-Empty or pCMV-Nanog construct (both at 0.3 μg/ml). Twenty-four hours after transfection, the cells were harvested and seeded at 800 per well in 96-well culture plates. For NT2/D1 cells, the cells were grown to 70% confluence in 60 mm culture plates.
Regulation of Nanog expression by protein kinase Cs

Six human cancer cell lines (NPC-076, HepG2, HT1376, SW620, T24 and Hep3B) were used to explore the possible effect of PKC activity on Nanog expression. The cell lines were incubated overnight with a broad-spectrum PKC inhibitor chelerythrine chloride, and the Nanog protein levels were examined by western blot analysis. As shown in Figure 1A, Nanog expression was induced by the suppression of PKC in these cell lines, the effect in NPC-076 and HepG2 cell lines was the most apparent and the effect in T24 and SW620 cell lines was the slightest. When the ratio of the Nanog to GAPDH in the untreated NPC-076 cells was set as 1, the ratio of chelerythrine chloride-treated cells was calculated to be 4.3 and 4.8, respectively, at a drug concentration of 5 and 10 μM. In HepG2 cells, treatment of the cells with chelerythrine chloride at 5 and 10 μM also resulted in the elevation of Nanog protein level by ~1.8- and 2.8-fold, respectively.

Nanog expression was further examined in a Nanog non-expressing human NPC cell line by incubating overnight with PKC inhibitors staurosporine or chelerythrine chloride. Nanog protein was found to be dose-dependently upregulated by ~4.7- and 6.7-fold in cells treated, respectively, with 5 and 10 μM of chelerythrine chloride (Figure 1B). Similarly, treatment of the cells with staurosporine at 10 and 20 μM also led to increased Nanog expression by ~1.9- and 3.7-fold, respectively compared with that of the control cell (Figure 1C). The stimulatory effect of PKC inhibitors on Nanog expression was further confirmed by semiquantitative RT–PCR. Consistently, Nanog mRNA levels were also found to be dose-dependently elevated by chelerythrine chloride or staurosporine treatment. Figure 1B shows that Nanog mRNA was increased by ~2.5- and 9.7-fold, respectively, in cells treated with 5 and 10 μM of chelerythrine chloride. Again, treatment of the cells with staurosporine at 10 and 20 μM also resulted in the elevation of Nanog mRNA by ~6.7- and 11.3-fold, respectively, compared with that of the untreated cell (Figure 1C).

Knockdown of PKCα/PKCδ enhances Nanog expression

To explore which PKC isoform is involved in the regulation of Nanog expression, siRNAs to PKCα, PKCδ and PKCe were...
designed to silence the respective PKC isoforms in NPC-076 cells. Figure 2A shows that suppression of PKCα or PKCδ expression upregulated Nanog expression when assayed by western blot analysis. Nanog protein levels were upregulated by ~72 and 31% in cells transfected, respectively, with siRNA to PKCα and PKCδ compared with that of the non-silencing siRNA-transfected cells. In contrast, NPC-076 cells expresses rather low level of PKCε and silencing of PKCε expression showed no obvious effect on Nanog expression.

To further confirm PKCδ is involved in the regulation of Nanog expression, rottlerin, a PKCδ-specific inhibitor, was used to treat NPC-076 and HepG2 cells (0, 5 and 15 μM), respectively. Nanog protein levels were found to be upregulated in NPC-076 and HepG2 cells on rottlerin exposure (Figure 2B).

PKC inhibitors stimulate Nanog promoter activity

We next examined the effect of the PKC inhibitor on the Nanog promoter activity. We transfected pGL3-Nanog-Luc into NPC-076 cells and examined the reporter activity with and without exposure to PKC inhibitor. As shown in Figure 3A, pGL3-Nanog-Luc reporter activity was dose-dependently stimulated by chelerythrine chloride. An increase of the reporter activity from 1.6- to 3.2-fold was observed when chelerythrine chloride was increased from 5 to 10 μM compared with that of the untreated cells.

Octamer–Sox composite element is involved in the PKC inhibitor-induced Nanog gene transcription

The effect of PKC activity on Oct4 and Sox2 protein levels was also examined by treating NPC-076 cells with chelerythrine chloride. Figure 3B shows that inhibition of PKC activity by chelerythrine chloride dose dependently increased Oct4 and Sox2 protein levels. Oct4 and Sox2 have been suggested to induce Nanog gene expression through Oct4–Sox2 complex formation (7). Here, we examined the effect of elevated Oct4 and Sox2 expression on the transcriptional activities of the pGL3-Nanog-Luc reporter. The transcriptional activities of the Nanog promoter were examined in NPC-076 cells by transfection with either pCMV-Oct4, pCMV-Sox2 or pCMV-Oct4 and pCMV-Sox2. Transfection of pCMV-Oct4 elicited 1.2-fold stimulation, transfection of pCMV-Sox2 elicited 1.4-fold stimulation, whereas transfection of pCMV-Oct4 and pCMV-Sox2 elicited a 3.2-fold stimulation of Nanog promoter activity compared with pCMV-Empty-transfected cells (Figure 3C). The result suggested that Oct4 acts together with Sox2 to positively regulate Nanog expression in NPC-076 cells on Nanog expression.

To further ascertain the involvement of Octamer–Sox composite element in PKC inhibition-induced transactivation, the conserved Octamer (TTTTGCAT) and Sox (TACAATG) elements between −166 and −180 of the Nanog promoter were mutated, and the reporter activities of pGL3-Nanog-Luc, pGL3-Nanog-mut-Oct4-Luc and pGL3-Nanog-mut-Sox2-Luc were examined (Figure 3D).
Regulation of Nanog expression by protein kinase Cs

Moreover, transfection of pCMV-PKCα, PMA ranging from 5 to 50 nM suppressed the luciferase activity in pGL3-Nanog-Luc-transfected cells. The luciferase activity in pGL3-Nanog-Luc reporter activities were compared in cells with and without PMA. As shown in Figure 5D, treatment of the NT2/D1 cells with PMA (5–50 nM) resulted in a dose-dependent reduction of Nanog transcript. In addition, treatment of PMA also downregulated the expression of miR302b-367 cluster, which were expressed at extremely high levels in human embryonal carcinoma cells and human ES cells and has been shown to gradually decreased on cell differentiation.

To examine the effect of PKCα activity on Nanog at protein level, we transfected a PKCα-expressing vectors, pCMV-PKCα, into NT2/D1 cells. We found that ectopically expressed PKCα elicited a repressive effect on Nanog expression on days 2 and 3 after transfection (Figure 5C). Moreover, transfection of pCMV-PKCα also suppressed expression of TRA-1–60, a heavily glycosylated membrane protein that is specifically expressed in undifferentiated pluripotent stem cells (28). The results suggested that elevated PKCα expression causes NT2/D1 cell differentiation and may downgrade its pluripotent status.

To examine the putative Octamer–Sox composite element of the Nanog promoter region is involved in PKC-suppressive effect, we examined the effect of PMA treatment on the transcriptional activities of the pGL3-Nanog-Luc, pGL3-Nanog-mut-Oct4-Luc and pGL3-Nanog-mut-Sox2-Luc luciferase reporter constructs in NT2/D1 cells. As shown in Figure 5D, pGL3-Nanog-Luc reporter activities were repressed by ~60% in cells treated with PMA (10–50 nM) compared with that of the untreated control cells. Mutation of the Octamer or Sox elements abolished the suppression of Nanog promoter by PMA compared with that of the wild-type (pGL3-Nanog-Luc) control. The results clearly suggested that the Octamer–Sox composite element is involved in the suppression of Nanog expression by PKC activity.

To further determine if PKC play a role in NT2/D1 cell differentiation, the cells were transfected with pCMV-Empty or pCMV-PKCα and cultured in the presence of G418 (500 μg/ml) for selection. Total protein from stably transfected clones were prepared and analyzed by western blotting for the expression of some differentiation markers. Nanog was found to be reduced dramatically in pCMV-PKCα-transfected cells (Figure 5E). Moreover, the stem cell marker TRA-1–60 was lost, involucrin—a protein precursor of the cross-linked envelope that is expressed in the terminally differentiated keratinocyte—was upregulated, and NeuroD1—a marker for neuronal differentiation—was increased.

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Fig. 5. Activation of PKC activity suppresses Nanog expression in NT2/D1 cells. (A) The NT2/D1 cells were incubated with PMA at the indicated concentrations. Twenty-four hours after incubation, the cells were harvested and the western blot analysis was performed using anti-Nanog, anti-Oct4, anti-Sox2 and anti-GAPDH antibodies. To compare the relative band intensities, all immunoreactive bands were normalized against GAPDH by densitometry. The ratio in the untreated cells was set as 1. Data are mean ± SD of three independent experiments. Significance is tested using one-way ANOVA with Holm–Sidak method. *P < 0.05 and **P < 0.01. (B) The NT2/D1 cells were treated with PMA at the indicated concentrations for 24 h and the expression of Nanog, Oct4, Sox2, miR302b-367 cluster and β-actin genes was assessed by RT–PCR as described in the Materials and methods. Amplified PCR products were run on a 1% agarose gel. The β-actin gene expression was used for loading control. Figures are representative of at least three independent experiments. (C) The NT2/D1 cells were transfected with either pCMV-Empty or pCMV-PKCα for 24, 48 and 72 h. Total cell lysates were prepared after treatment and were subjected to western blot analysis with anti-Nanog, anti-PKCα, anti-TRA-1–60 and anti-GAPDH antibodies. To compare the relative band intensities, all immunoreactive bands were normalized against GAPDH by densitometry. The ratio in the untreated cells or pCMV-Empty-transfected cells was set as 1. Data are mean ± SD of three independent experiments. The relative band intensities of the cells transfected with PKCα were compared with that of the control using t-test. **P < 0.01 is considered as statistically significant. (D) The NT2/D1 cells were transfected with either wild-type pGL3-Nanog-Luc, pGL3-Nanog-mut-Oct4-Luc or pGL3-Nanog-mut-Sox2-Luc for 24 h. The cells were also cotransfected with pSV-β-galactosidase vector, and the β-galactosidase activity was used to normalize the luciferase activity. The cells were treated with increasing concentrations of PMA for 24 h after transfection. Luciferase activity is presented as the fold induction relative to PMA-untreated cells. Data are mean ± SD from triplicate analysis. Asterisks indicate a significant difference compared with the wild-type control. *P < 0.05 (t-test). (E) The NT2/D1 cells were stably transfected with either pCMV-Empty or pCMV-PKCα for 3 weeks. Total cell lysates were prepared and subjected to western blotting analysis with anti-Nanog, anti-PKCα, anti-TRA-1–60, anti-involucrin, anti-NeuroD1 and anti-GAPDH antibodies. To compare the relative band intensities, all immunoreactive bands were normalized against GAPDH by densitometry. The ratio in the untreated cells or pCMV-Empty-transfected cells was set as 1.

pGL3-Nanog-mut-Sox2-Luc were compared in cells with and without chelerythrine chloride treatment. As shown in Figure 3D, mutation of the Octamer–Sox composite element attenuated the upregulation effect of chelerythrine chloride on Nanog expression. To functionally test this promoter region, we transfected NT2/D1 cells with pGL3-Nanog-Luc containing −993/+231 region of the human Nanog gene to drive the luciferase expression. As shown in Figure 3E, the luciferase activity in pGL3-Nanog-Luc-transfected cells was 23.6-fold higher than the pGL3-basic- (a promoter-null control) transfected cells. To examine the functional significance of the Octamer–Sox composite element for Nanog promoter activity, the cells were transfected with Nanog reporter construct containing substitution mutation at either Oct4 element or Sox2 element (pGL3-Nanog-mut-Oct4-Luc and pGL3-Nanog-mut-Sox2-Luc), and the reporter activity was examined. As anticipated, both mutations reduced promoter activity by 4.6- and 3.1-fold, respectively (Figure 3E).

**Knockdown of Oct4 or Sox2 suppresses the PKC inhibitor-induced Nanog expression**

The possible involvement of Oct4 and Sox2 in PKC inhibitor-induced Nanog gene transcription was further studied by knockdown of Oct4 or Sox2 using respective siRNA. As shown in Figure 4, transfection of NPC-076 cells with siRNA against either Oct4 or Sox2 blocked chelerythrine chloride-induced Nanog expression at protein level. The results showed that expression of Oct4 and Sox2 is essential for the PKC inhibitor-induced Nanog expression.

**Activation of PKC suppresses Nanog and its target genes expression in Nanog-expressing cells**

Nanog-expressing cells (NT2/D1 and NCCIT cells) were incubated overnight with increasing concentrations of PKC activator PMA. As shown in Figure 5A, PMA ranging from 5 to 50 nM suppressed endogenous Nanog protein levels compared with that of the untreated cells. Treatment with PMA also suppressed the expression levels of Oct4 and Sox2, the transcription factors that were believed to positively regulate Nanog expression. To explore the possible mechanism of PKC-induced downregulation of Nanog, we examined whether the reduced Nanog protein is due to a reduced Nanog mRNA level. As shown in Figure 5B, treatment of the NT2/D1 cells with PMA (5–50 nM) resulted in a dose-dependent reduction of Nanog transcript. In addition, treatment of PMA also downregulated the expression of miR302b-367 cluster, which were expressed at extremely high levels in human embryonal carcinoma cells and human ES cells and has been shown to gradually decreased on cell differentiation.

To examine the effect of PKCα activity on Nanog at protein level, we transfected a PKCα-expressing vectors, pCMV-PKCα, into NT2/D1 cells. We found that ectopically expressed PKCα elicited a repressive effect on Nanog expression on days 2 and 3 after transfection (Figure 5C). Moreover, transfection of pCMV-PKCα also suppressed expression of TRA-1–60, a heavily glycosylated membrane protein that is specifically expressed in undifferentiated pluripotent stem cells (28). The results suggested that elevated PKCα expression causes NT2/D1 cell differentiation and may downgrade its pluripotent status.

To examine if the putative Nanog-expressing cells and were subjected to western blot analysis with anti-Nanog, anti-PKCα, anti-TRA-1–60, anti-involucrin, anti-NeuroD1 and anti-GAPDH antibodies. To compare the relative band intensities, all immunoreactive bands were normalized against GAPDH by densitometry. The ratio in the untreated cells or pCMV-Empty-transfected cells was set as 1.
Fig. 6. Nanog expression promotes cell proliferation of human cancer cell lines. (A) The NPC-076 cells were transfected with pCMV-Empty vector (0.3 μg/ml) or pCMV-Nanog vector (0.3 μg/ml). In contrast, the NT2/D1 cells were transfected with siRNA (50nM) against Nanog or with non-silencing control. The transfected cells were plated at 800 cells per 60 mm dish and were cultured for 10 days. Colonies were fixed and stained with crystal violet. Dishes were photographed as shown in upper panel (scale bar = 10 mm). Colonies were measured using Image J software (National Institutes of Health). Colonies with an area ≥1.5 mm² were scored and bar graphed. Data computed from non-silencing siRNA-transfected NT2/D1 cells or pCMV-Empty-transfected NPC-076 cells were set as 100%. The colony covered area in each plate was measured as described. Data are expressed as the mean value ± SD of three different experiments. Significance was tested using one-way ANOVA with Holm–Sidak method post hoc test, where *P < 0.05 and **P < 0.01. (B) Ectopic Nanog expression
Ectopic expression of Nanog increases cell proliferation and colony formation in Nanog non-expressing cells

We have thus far shown that PKC activity regulates Nanog expression in human cancer cell lines. However, the cell biological effect of Nanog expression in these cells is still unclear. Here, we employed cell colony-forming assay to examine whether Nanog expression has any effect on cell proliferation. The NPC-076 cells were transfected with pCMV-Empty or pCMV-Nanog construct, plated at 800 cells per 60 mm dish, and colonies formed were counted on 10th day after initial plating. To avoid the recognition of cell debris, background staining or other artifacts as ‘colonies’ in the counting procedure, images were acquired with a scanner and all colonies >1.5 mm in diameter were counted. As shown in Figure 6A, ectopic expression of Nanog led to a conspicuously better cell growth compared with the Nanog-unexpressed control. The area covered by the colonies in pCMV-Empty-transfected culture was measured using Image J software and was set as 100%. Accordingly, the colony covered area in pCMV-Nanog-transfected cells was calculated to be 258% ± 35 (Figure 6A).

The growth-promoting effect of Nanog expression was further confirmed by MTT assay. The NPC-076 cells were transfected with pCMV-Empty or pCMV-Nanog and the cell growth was assessed by MTT assay. The cell proliferation rate was measured everyday from day 1 to day 4. The data showed that cell proliferation was significantly higher in pCMV-Nanog-transfected cells than in the control groups (Figure 6B).

Knockdown of Nanog suppresses cell proliferation and colony-forming efficiency

The NT2/D1 cells are constitutive with Nanog expression. To examine the possible role of Nanog in cell proliferation, we transfected NT2/D1 cells with siRNA against Nanog or non-silencing siRNA and compared their colony-forming efficiency. As shown in Figure 6A, both colony size and number were markedly reduced in Nanog siRNA-transfected cultures than the non-silencing control cultures. Silencing of Nanog by siRNA led to a 79% decrease of the colony covered area compared with the non-silenced culture.

The MTT assay was used to compare the cell growth rate between the two groups. The result showed that cell proliferation of the Nanog siRNA-transfected cells was significantly reduced compared with the non-silencing siRNA-transfected cells (Figure 6B). Taken together, our results suggested that Nanog plays an important role in regulating cell proliferation in human cancer cell lines regardless of their Nanog expression status.

Discussion

ES cells are capable of self-renewing and maintain in a proliferative state for prolonged period of time and are capable of differentiating into many specialized cell types (29). Several extrinsic growth factors have been found to support the pluripotency of ES cells. For examples, leukemia inhibitory factor (LIF) was shown to support the pluripotent state of the cultured mouse ES cells by activating Signal transducer and activator of transcription 3 (STAT3) (30). Bone morphogenetic protein 4 (BMP4) was shown to support mouse ES cell self-renewal. Together, LIF and BMP4 were shown to enhance self-renewing and pluripotency of mouse ES cells by activating Id genes (inhibitor of differentiation) family (31). Intriguingly, LIF alone was not sufficient to maintain human ES cells and BMPs caused rapid differentiation (32,33). Instead, fibroblast growth factor signaling pathway and a balance between transforming growth factor-β/activin and BMP signaling pathways were shown to be important in the maintenance of self-renewing of human ES cells (34–37). Functionally, these extrinsic regulators ultimately led to gene expression that regulate the pluripotent state.

The best-characterized genes of these are a number of transcription factors, such as Oct4, Sox2 and Nanog (1). Nanog has been shown to be a key factor in maintaining ES cell pluripotency (34,35), it has been further suggested to function in concert with Oct4 and Sox2 to maintain the pluripotency and self-renewing characteristics of human ES cells (38). In addition to be expressed in stem cells, Nanog has also been shown to express in various human tumor cells (9–15). Thus, the regulation of the Nanog gene expression is likely to be an important event in maintaining stem cell pluripotency and perhaps, in tumorigenesis as well.

Theoretically, a reduced Nanog activity is required to promote differentiation in embryonic development. During ES cell differentiation, rapid downregulation of Nanog was shown to correlate with Ser315 phosphorylation of p53, which led to an increase of its transcriptional regulation activity (39). However, when p53-es ES cells were induced to differentiate by retinoic acid, Nanog was still downregulated (39), suggesting the existence of other negative regulator for Nanog expression during ES cell differentiation. PKC activity has been shown to be upregulated during the differentiation of human embryonal carcinoma cell line NT2/D1 (22,24). We, therefore, thought that PKC may be a candidate regulator for Nanog expression that influences ES cell fate.

Indeed, here we showed that Nanog expression was suppressed by increased PKC activity. We found that in Nanog-expressing cell, the expression levels of Nanog and its downstream genes were suppressed by PMA or ectopically expressed PKCα. In addition, two positive regulators of Nanog expression, Oct4 and Sox2, were also downregulated by PMA treatment. Activation of PKC may in turn phosphorilate transcription factors and thereby stimulate gene transcription by binding to specific response elements in the promoter region of responsive genes that are involved in the regulation of cellular responses such as proliferation and differentiation. In general, gene expression is initiated 12 h after desired regulator transfection (40), thus, it is reasonable to see that maximal effect by the ectopic expression of PKCα is observed after 2 days. Phosphorylation of many transcription factors by activation of PKC is a crucial signal transduction event that results in an altered expression pattern of many genes. The influence on gene expression is mediated by transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B. Previous report indicated that the half-life of AP-1 activity is estimated to be 6 h (41). The residual AP-1 activity could be from a preexisting Fos/Jun heterodimer or Jun/Jun homodimer. To avoid such interference, the Nanog-non-expressing cell was incubated overnight with PKC inhibitors in our study. In Nanog-non-expressing cell, Nanog was dose-dependently induced by treatment with PKC inhibitors. In Figure 1B, the enhancement of Nanog protein level was much stronger than mRNA level at 5 μM chelerythrine chloride. Currently, it is unclear why there is such discrepancy; one possible explanation is that chelerythrine chloride some how stabilizes Nanog protein. To determine the stability of Nanog protein by PKC signaling pathway, the NT2/D1 cells were incubated with cycloheximide in the presence or absence of PMA. The result showed that the treatment of PMA did not significantly accelerate the decline of Nanog protein (Supplementary Figure 1, available at Carcinogenesis Online). We, thus, suggested that the difference of mRNA level and protein level of Nanog is due to the detection sensitivity of the experimental methods employed.

To examine which PKC isoform was responsible for the suppression of Nanog expression, NPC-076 cells were treated with siRNA against selected PKC isoforms and Nanog gene expression was examined. We found that Nanog expression was dramatically increased by knockdown of PKCα or PKCd, suggesting that blockade of PKC expression is a positive signal for Nanog expression in Nanog non-expressing human cancer cell line, NPC-076.

We have thus far clearly showed that PKC inhibitors enhance Nanog expression in human cancer cell lines. Ectopic expression of

*promotes cell proliferation in NPC-076 cells. Cells were transfected with pCMV-Empty or pCMV-Nanog. Cell growth was analyzed by MTT assay at 24, 48, 72 and 96 h following transfection. The growth of pCMV-Empty-transfected cells were measured accordingly and served as a parallel control (left panel). Knockdown of Nanog suppresses cell proliferation in NT2/D1 cells. Cell proliferation after transfection with siNon or siNanog was assessed using MTT assay (right panel). Data are means ± SD from triplicate analysis. Asterisks indicate a significant difference compared with the control. *P < 0.05 and **P < 0.01 (t-test).
Oct4 and Sox2 are two positive regulators of Nanog expression, we, therefore, examined if Oct4 and Sox2 were also upregulated by reduced PKC activities. Thus, upregulation of Nanog by PKC inhibitor was coincided with increased expression of Oct4 and Sox2. Moreover, site-specific mutation of the Octamer–Sox composite element in Nanog promoter constructs abolished the stimulation of promoter activity by PKC inhibitors. Consistently, silence of Oct4 and Sox2 by siRNA attenuated PKC inhibitor-induced upregulation of Nanog expression. These studies strongly suggested that Oct4-Sox2 complexes were involved in PKC-regulated Nanog expression.

The cancer stem cell model of tumor development and progression infers that tumors contain a minority population of cells that can both maintain tumor growth and give rise to phenotypically diverse cancer cells. Recent reports indicated that cancer stem cells express normal stem cell characteristics and might thus harbor unique signaling pathways that maintain the undifferentiated state of normal stem cells (42). More recently, it has been shown that some aggressive cancers and cancer stem cells display gene expression signatures characteristic of ES cells (43,44). Oct4 and Sox2 were suggested to be essential transcription regulators for maintaining the pluripotency and self-renewing characteristics of human ES cells and have been found to be expressed in various cancer cells (11,45–50). In addition, clinical studies showed that Oct4 or Sox2 expression is associated with poor prognosis in bladder cancer, esophageal squamous cell carcinoma, hepatocellular carcinoma, oral squamous cell carcinoma and rectal cancer (45–49). Oct4 was suggested to play a key role in maintaining the survival of cancer cells in that knockdown of Oct4 in somatic tumor cells was shown to lead to tumor cell apoptosis and inhibition of tumor growth (51). Moreover, continuous Oct4 expression in epithelial tissues appeared to associate with dysplastic disorders by inhibiting cellular differentiation in a manner similar to that in ES cells (52). These observations suggested a role of PKC-Oct4/Sox2-Nanog pathway in tumor development. PKC activation has been shown to lead to AP-1 activation (53), it is, therefore, possible that the activated AP-1 family proteins could then exert their effect on the transcription of Oct4 and Sox2. However, how reduced PKC activity upregulated Oct4 and Sox2 expression is currently unclear. The involvement of microRNA in the regulation of Oct4 and Sox2 expression has also been reported. MicroRNAs mir-134 and mir-145 were shown to express in low levels in self-renewing ES cells but were highly expressed during differentiation (54–56). Xu et al. (56), showed that the pluripotency factors Oct4, Sox2 and Klf4 are direct targets of miR-145 and that endogenous mir-145 repressed the 3′ untranslated regions of Oct4, Sox2 and Klf4. Thus, it is possible that downregulation of Nanog by increased PKC activity may be regulated through the upregulation of some microRNA specie(s) in NT2/D1 cells.

Our study indicated that PKC activity plays a negative regulatory role on Nanog expression and could thus regulate the proliferation of cancer cells. Judging from the roles of Nanog expression in maintaining the pluripotency and self-renewing characteristics of human ES cells, PKCα and PKCδ are likely to play roles in embryonal development.

Supplementary material
Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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