FEBS Letters 586 (2012) 3529–3535

journal homepage: www.FEBSLetters.org

Regulation of human growth and differentiation factor 3 gene expression by NANOG in human embryonic carcinoma NCCIT cells

Sung-Won Park [a,1], Hye-Young Lim [b,1], Hyun-Jin Do [a], BoReum Sung [a], Sun-Hyung Huh [a], Sang-Jun Uhm [c], Hyuk Song [d], Hak-Jae Chung [e], Jin-Hoi Kim [b], Nam-Hyung Kim [f], Jae-Hwan Kim [a,*]

[a] Department of Biomedical Science, College of Life Science, CHA University, Seongnam-si, Gyeonggi-Do, South Korea
[b] Department of Animal Biotechnology, College of Animal Bioscience & Technology, Konkuk University, Seoul, South Korea
[c] Department of Animal Science & Biotechnology, Sangji Youngseo College, Wonju, Gwangwon-Do, South Korea
[d] Department of Animal & Food Bioscience, College of Natural Science, Konkuk University, ChungJu, Chungbuk, South Korea
[e] Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, Suwon, Gyeonggi-Do, South Korea
[f] Department of Animal Sciences, Chungbuk National University, Cheongju, Chungbuk, South Korea

ARTICLE INFO

Article history:
Received 11 May 2012
Revised 9 August 2012
Accepted 13 August 2012
Available online 19 August 2012

Edited by Ned Mantei

Keywords:
GDF3
NANOG
Tumorigenesis
Stem cell transcription factor
Oncogenic growth factor
Embryonic carcinoma NCCIT cell

ABSTRACT

We investigated transactivation by NANOG in regulating growth and differentiation factor 3 (GDF3) expression in NCCIT cells. GDF3 expression was affected by shRNA-mediated downregulation and by exogenous overexpression of NANOG specifically, as well as by retinoic acid-mediated differentiation. GDF3 transcription was activated by NANOG, and the upstream region (−183 to −1) was sufficient to induce minimal transcriptional activity. Moreover, NANOG binds to the GDF3 minimal promoter in vivo and the transcriptional activity is mediated by NANOG transactivation domain. This study provides the first evidence that NANOG is a transcriptional activator of the expression of the oncogenic growth factor GDF3 in embryonic carcinoma cells.

1. Introduction

Pluripotent stem cells possess the unique capabilities of self-renewal, proliferation, and differentiation into multiple cell-types. Coordinated transcription factor networks including NANOG, OCT4, and SOX2 have emerged as master regulators of stem cell pluripotency and differentiation [1]. Germ cell tumors (GCTs), which arise from primordial germ cells, contain undifferentiated and pluripotent embryonic carcinoma (EC) cells and exhibit a gene expression profile similar to that of pluripotent stem cells, including expression of the transcription factors NANOG, OCT4, and SOX2 [2]. It has been suggested that deregulation of these networks and factors may contribute to malignant transformation [3–5]. Especially, NANOG is a novel homeobox-containing transcription factor and a key regulator of embryonic stem cell self-renewal and pluripotency [6,7]. NANOG is expressed not only in GCTs, but also in other tumors including carcinomas of the breast, cervix, oral cavity, kidney, and ovary [8–14]. Moreover, ectopic overexpression of NANOG induced the proliferation and transformation of NIH3T3 and 293T cells; NANOG has also been demonstrated to regulate human tumor development [15–17].

Growth and differentiation factor 3 (GDF3) belongs to the bone morphogenic protein/GDF class of the transforming growth factor β (TGF-β) superfamily [18]. Human GDF3 was first identified in human carcinoma cells and, subsequently, in primary testicular germ cell tumors (TGCTs), seminomas, and breast carcinomas [10,19,20]. In normal tissues, GDF3 is expressed in embryonic stem cells and the early embryo [21,22]. GDF3-null mice exhibit developmental abnormalities, suggesting that GDF3 function is required during embryonic development [19]. Recent work has demonstrated that GDF3 can induce progression of B16 cell melanomas, suggesting a possible role in tumor progression [23]. Despite sharing a similar expression pattern in cancers as well as in pluripotent stem cells, there is no evidence that NANOG and GDF3 interact in regulating gene expression at the transcriptional level.

Abbreviations: GDF3, growth and differentiation factor 3; GCT, germ cell tumor; TGCT, testicular germ cell tumor

* Corresponding author. Address: Department of Biomedical Science, College of Life Science, CHA University, 222 Yatap-Dong, Bundang-Gu, Seongnam-Si, Gyeonggi-Do 463-836, South Korea. Fax: +82 31 725 8350.
E-mail address: jaehwan_k@cha.ac.kr (J.-H. Kim).

[1] These authors contributed equally to this work.

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.08.013

We hypothesize that the stem cell transcription factor NANOG regulates GDF3 gene expression in NCCIT cells, which have a phenotype intermediate between seminoma and EC cells and are a useful tool for studying the relationship between seminoma and non-seminoma tumorigenesis [24]. This study provides the first evidence that NANOG is a transcriptional activator of the expression of the oncogenic growth factor GDF3 in EC cells and further contributes to our understanding of the molecular mechanisms by which stem cell factors mediate tumorigenesis and pluripotency processes.

2. Materials and methods

2.1. Plasmid construction

The luciferase reporter constructs that contain regions upstream of the human GDF3 promoter were generated as follows and designated as -1721-Luc and -183-Luc. The human GDF3 promoter region (−1721 to −1; nucleotide position relative to the translational start site as +1) was amplified by PCR using genomic DNA from NCCIT cells and cloned into the pGL3-basic reporter plasmid (Promega, Madison, WI, USA). A series of deletion mutants, in which nucleotides downstream of positions −1339, −1113, −758, −536, −254, and −183 to −1 from the translation start site of the GDF3 gene were contained, were generated using the full-length human GDF3 promoter construct as a template. Mutant promoter constructs were produced from the GDF3 minimal promoter region (−183 to −1) using the QuickChange Site-Directed Mutagenesis method (Stratagene, La Jolla, CA, USA). Human NANOG and deletion variants were generated using full-length hNANOG cDNA isolated from NCCIT cells. The constructs were inserted into the pcDNA3.1 plasmid in frame with a Flag epitope tag. All cloned PCR products and reporter plasmids were verified by sequencing.

2.2. Cell culture and differentiation

NCCIT and HEK293T cells (American Type Cell Collection) were grown in Dulbecco's Modified Eagle's Medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). To induce differentiation, NCCIT cells were treated with 10 μM retinoic acid (RA, Sigma, St. Louis, MO, USA) for 10 days.

2.3. Preparation of short hairpin RNA against hNANOG

Target sequence for hNANOG RNA interference was obtained from the previous report [25] for maximum silencing efficiency. Two double-stranded oligonucleotides were generated by annealing 5′-GATCCGCCAGACCTGGAACAATTCA*GAAGCTTG*TGAATTGTTCCAGGTCTGGTTTTTTGGAAGC-3′ and 5′-GGCCGCTTCCAAAAAACCAGACCTGGAACAATTCA*CAAGCTTC*TGAATTGTTCCAGGTCTGGCG-3′ and inserted into *Bam*HI and *Not*I-digested pGSH1-GFP shRNA vector (Genlantis, San Diego, CA, USA). NANOG sense and antisense sequences are underlined. The hairpin loop structure is underlined and the *Hind*III sequence is marked in italics. The pGSH1-GFP-luciferase shRNA vector used for a control was provided by the manufacturer.

2.4. Microporation

NCCIT cells were transfected with each shRNA expression vector or wild-type NANOG expression vector by using a microporator (1100 volts, 20 ms; Digital Bio Tech., South Korea) and collected 2 days post-transfection for real-time PCR and Western blot analyses.

2.5. RNA preparation and real-time PCR

Total RNA was prepared from undifferentiated and differentiated NCCIT cells and subjected to reverse transcription using 5 μg total RNA, oligo dT primers (Promega), and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Grand Island, NY, USA). The cDNAs were amplified with real-time PCR mix containing the DNA binding dye SYBR green (Bioneer, Daejeon, South Korea). The forward and reverse primers for quantitative real-time RT-PCR were as follows: GDF3 (150 bp), 5′- AGACTTATGCTACGTAAAGGAGCT -3′ and 5′- CTTTGATGGCAGACAGGTTAAAGTA -3′; NANOG (107 bp), 5′- CCCAAAGGCAAACAACCCACTTCT -3′ and 5′- AGCTGGGTGGAAGAGAACACAGTT -3′; and GAPDH (226 bp), 5′- GAAGGTGAAGGTCGGAGTC -3′ and 5′- GAAGATGGTGATGGGATTTC -3′. GAPDH cDNA was amplified from all samples as a normalizing control. Relative quantification of the expression levels was determined using the 2-ΔΔCT method [26].

2.6. Western blot analysis

NCCIT cells transfected with NANOG shRNA or Flag-tagged NANOG expression vector were harvested 48 h after transfection. For Western blot analysis, cells were lysed with 1 ml of RIPA buffer containing 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), and 50 μM MG-132 proteasome inhibitor (EMD Millipore, Billerica, MA, USA) on ice for 20 min. Whole cell lysates were separated by 10% SDS–polyacrylamide gel electrophoresis and proteins were transferred to a PVDF membrane (GE Healthcare, Waukesha, WI, USA). Membranes were blocked in 5% non-fat milk and then incubated with an anti-Flag monoclonal (1:2500, Sigma), anti-NANOG polyclonal (1:2500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GDF3 polyclonal (1:1000, Abcam, Cambridge, MA, USA), and anti-β-actin monoclonal (1:2500, Santa Cruz Biotechnology) antibodies, followed by incubation with a horseradish peroxidase-conjugated secondary antibodes (1:5000, Santa Cruz Biotechnology). Immunoreactive proteins were visualized using an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL, USA).

2.7. Transient transfection and reporter assays

NCCIT and HEK 293T cells were used for transient transfection and reporter assays were performed in duplicate in three or more independent experiments, as described in previous reports [27,28].

2.8. Chromatin immunoprecipitation

Naïve NCCIT cells were fixed in 1% formaldehyde for 10 min at room temperature, resuspended in 1 ml of swelling buffer (100 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP40, 1 mM DTT and protease inhibitors) and incubated for 30 min on ice. After centrifugation at 4 °C for 10 min, nuclei were resuspended in 1.5 ml of sonication buffer (50 mM Tris, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% SDC, 0.1% SDS and protease inhibitors) and sonicated 8 times for 20 s, resulting in an average fragment size of 1000 bps. Chromatin was precleared with salmon sperm DNA and protein G-plus agarose beads (Santa Cruz) for 4 h at 4 °C. Precleared chromatin (1 ml) was rotated overnight at 4 °C with 3 μl of the rabbit monoclonal anti-NANOG antibody (Cell Signaling) or normal rabbit IgG (Cell Signaling). Chromatin/antibody complexes were incubated with 50 μl of Protein G-plus agarose beads for 6 h. Agarose beads were washed with immune complex wash buffers in the following order: low salt (20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, 0.1% SDS), high salt (20 mM Tris, (pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% TritonX-100, 0.1% SDS), LiCl (10 mM Tris, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 1%