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Nanog positively regulates Zfp57 expression in mouse embryonic stem cells



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ABSTRACT

To maintain the self-renewal of embryonic stem (ES) cells, several core transcription factors, including Oct3/4, STAT3, and Nanog, regulate the expression of their target genes. Zinc finger protein 57 (Zfp57) is specifically expressed in self-renewing ES cells and its expression level is reduced upon ES cell differentiation, suggesting that expression of this transcription factor is regulated by core transcription factors. In the present study, we investigated whether Zfp57 expression is regulated by Nanog. Nanog overexpression resulted in the upregulation of Zfp57. On the other hand, knockdown of Nanog reduced the expression level of Zfp57. In addition, we identified the Nanog-responsive region in the promoter of the *Zfp57* gene. These results suggest that Nanog is an upstream regulator of Zfp57. Moreover, Nanog overexpression promoted the growth of ES cells in soft agar and this was suppressed by Zfp57 knockdown, suggesting that the Nanog/Zfp57 pathway plays a central role in anchorage-independent growth of ES cells. Interestingly, NANOG overexpression also led to the upregulation of ZFP57 in two human tumor cell lines. Taken together, our results suggest that Nanog positively regulates Zfp57 expression in multiple types of cells.

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1. Introduction

Embryonic stem (ES) cells are pluripotent stem cells that are established from the inner cell mass of blastocysts. In the presence of leukemia inhibitory factor (LIF), mouse ES cells remain in the undifferentiated state and continue to self-renew indefinitely. Extensive studies have revealed the molecular mechanism underlying the regulation of ES cell self-renewal [1]. For example, it is well-established that several transcription factors, such as Oct3/4 [2,3], Nanog [4,5], and STAT3 [6,7], play central roles in the maintenance of self-renewal via the formation of core molecular networks [8,9].

Zinc finger protein 57 (Zfp57) is a transcription factor with several zinc finger motifs and a Krüppel-associated box (KRAB) domain. This transcription factor was originally cloned from a mouse teratocarcinoma cell line as an undifferentiated cell-specific gene [10]. Functional analysis using knockout mice revealed that loss of the zygotic function of Zfp57 leads to partial lethality, while eliminating both the maternal and zygotic functions of Zfp57 results in complete embryonic lethality [11]. The ZFP57 protein recruits KRAB-associated protein 1 (KAP1), a scaffold molecule for heterochromatin-inducing factors, to multiple imprinting control regions, and thus participates in regulation of genome imprinting [11–13]. Although the successful establishment of *Zfp57*-null ES cells indicates that Zfp57 is dispensable for the maintenance of ES cell self-renewal [14], we recently reported that this transcription factor is involved in the anchorage-independent growth of ES cells [15]. In addition, ZFP57 can stimulate anchorage-independent growth of the mouse immortal fibroblast cell line NIH3T3 and promote tumor formation of human fibrosarcoma HT1080 cells in nude mice. Furthermore, our immunohistochemical analysis revealed that ZFP57 is overexpressed in several tumor tissues. Similarly, Cirillo et al. recently showed that increased expression

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of ZFP57 is associated with high-grade glioblastoma [16]. These findings suggest that ZFP57 can function as an oncogene in some types of cancer. In addition, mutations in the ZFP57 gene can reportedly cause transient neonatal diabetes mellitus type 1 [17].

Among mouse ES cells, expression of Zfp57 is restricted to those cells in an undifferentiated state and is lost following LIF removal [14], suggesting that expression of Zfp57 is regulated by self-renewal-related transcription factors. Zfp57 is a downstream molecule of STAT3 and Oct3/4 [14]. However, the possible involvement of Nanog, another self-renewal transcription factor, in Zfp57 regulation has not been investigated. In this study, we explored this possibility and found that Nanog also regulates Zfp57 expression in mouse ES cells. In addition, we found that activation of the Nanog/Zfp57 axis promotes anchorage-independent growth of ES cells and that NANOG also regulates ZFP57 expression in human tumor cells.

2. Materials and methods

2.1. Cell culture

The mouse ES cell line E14 (E14tg2a) and the human colorectal adenocarcinoma cell line HT29 were obtained from the American Type Culture Collection. The human fibrosarcoma cell line HT1080 was obtained from the Health Science Research Resources Bank (Osaka, Japan). E14 and HT1080 cells were cultured as described previously [15]. HT29 cells were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France).

2.2. Plasmid construction

Construction of the mammalian expression vectors, pCAG-IP and pCAGIP-myc-Nanog, has been described previously [18]. A DNA fragment containing emerald green fluorescent protein (EmGFP) cDNA and control microRNA (miRNA) was transferred from pGW/EmGFP-control miRNA [15] to pCAG-IP and pCAG-IPH3 [15] to obtain pCAGIP-EmGFP-control miRNA and pCAGIPH3-EmGFP-control miRNA, respectively. The coding region of myc-tagged human NANOG was inserted into pEBM-CAGIP [15] to obtain pEBM-CAGIP-myc-hNanog. Expression vectors for artificial miRNAs against Nanog, namely, pCAGIP-EmGFP-Nanog(582) and -Nanog(710), were constructed by replacing the miRNA of pCAGIP-EmGFP-control miRNA with 5'-ctg TTA TAG CTC AGG TTC AGA ATG GTT TTG GCC ACT GAC TGA CCA TTC TGA CTG AGC TAT AAc ag-3' and 5'-ctg AAT GGA TGC TGG GAT ACT CCA GTT TTG GCC ACT GAC TGA CTG GAG TAT CAG CAT CCA Ttc ag-3', respectively. Similarly, expression vectors for artificial miRNA against Zfp57, namely, pCAGIPH3-EmGFP-Zfp57(1008) and -Zfp57(1082), were obtained by replacing the miRNA of pCAGIPH3-EmGFP-control miRNA with 5'-ctg TAA ATG AGC ACG TCG GTG ACG GTT TTG GCC ACT GAC TGA CCG TCA CCG GTG CTC ATT TAc ag-3' and 5'-ctg TTC AGG TGA CGG TTG ACC TGA GTT TTG GCC ACT GAC TGA CTG AGG TCA CGT CAC CTG AAc ag-3', respectively. Using genomic DNA isolated from the mouse ES cell line A3-1 [19] as a template, a 289-bp fragment of the Zfp57 promoter (positions -210 to +79) was amplified by PCR with the primers 5'-GGT ACC AAG GAA TTA CGG AAA TTG AGT C-3' and 5'-CTC GAG AAG TCA GGA ATC CGC TAC TTT G-3'. The amplified fragment was then inserted into pGL4.10 (Promega, Madison, WI) to obtain pGL4-zfp57 (-210/+79).

2.3. RT-PCR analysis

Total RNA was isolated with Sepasol-RNA I Super G (Nacalai Tesque) and converted to cDNA using Revertra Ace (Toyobo, Osaka,

Japan) and oligo(dT)₁₂₋₁₈ primers (Nippon Gene, Tokyo, Japan). The primer sets used for PCR analysis are listed in [Supplementary Table S1](#). Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as an internal control.

2.4. Reporter assay and soft agar assay

For the reporter assay, cells were cultured for 2 days after transfection and then harvested to prepare a cell lysate. The luciferase activity of each lysate was measured using a dual luciferase assay system (Promega) according to the manufacturer's protocol.

The soft agar assay was basically performed as described previously [15]. Briefly, 300 cells were seeded into culture medium containing 0.5% agarose in 6-cm Petri dishes (Kord Products, Ontario, Canada). Cells were then cultured for 1–2 weeks and viable colonies were stained with 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Nacalai Tesque).

3. Results

3.1. Nanog upregulates Zfp57 expression in ES cells

To determine if Nanog is an upstream regulator of Zfp57 in mouse ES cells, we first examined the effect of Nanog overexpression on the expression level of Zfp57. When Nanog was overexpressed in ES cells, expression of Zfp57 was upregulated ([Fig. 1A](#)), suggesting that Zfp57 is a downstream molecule of Nanog. To confirm this, we suppressed Nanog expression using two artificial microRNAs (miRNAs) ([Supplementary Fig. S1](#)). Nanog knockdown resulted in the downregulation of Zfp57 ([Fig. 1B](#)), which corresponds well with the results of a previous microarray analysis [20]. These results suggest that Nanog positively regulates Zfp57 expression.

To search for a Nanog-responsive region in the Zfp57 gene, we preliminarily cloned several regions from 0 to 6 kb upstream of the transcription start site and measured their enhancer/promoter activities. A region of approximately 300 bp (−210 to +79) exhibited high promoter activity ([Fig. 2A](#)). Furthermore, Nanog overexpression increased the promoter activity of this region ([Fig. 2B](#) and [Supplementary Fig. S2](#)), while Nanog knockdown decreased the promoter activity of this region ([Fig. 2C](#)). These results suggest that this region is involved in Nanog-stimulated Zfp57 expression.

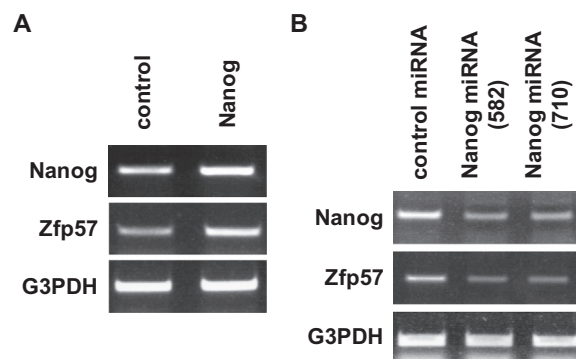


Fig. 1. Nanog upregulates Zfp57 expression in ES cells. (A) Overexpression of Nanog leads to upregulation of Zfp57. E14 cells were transfected with Nanog and subjected to RT-PCR analysis using the primers listed in [Table S1](#). (B) Nanog knockdown results in downregulation of Zfp57. After transfection with an expression vector for negative control miRNA, Nanog miRNA(582), or Nanog miRNA(710), E14 cells were subjected to RT-PCR analysis. In both panels, data are representative of three independent experiments. G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

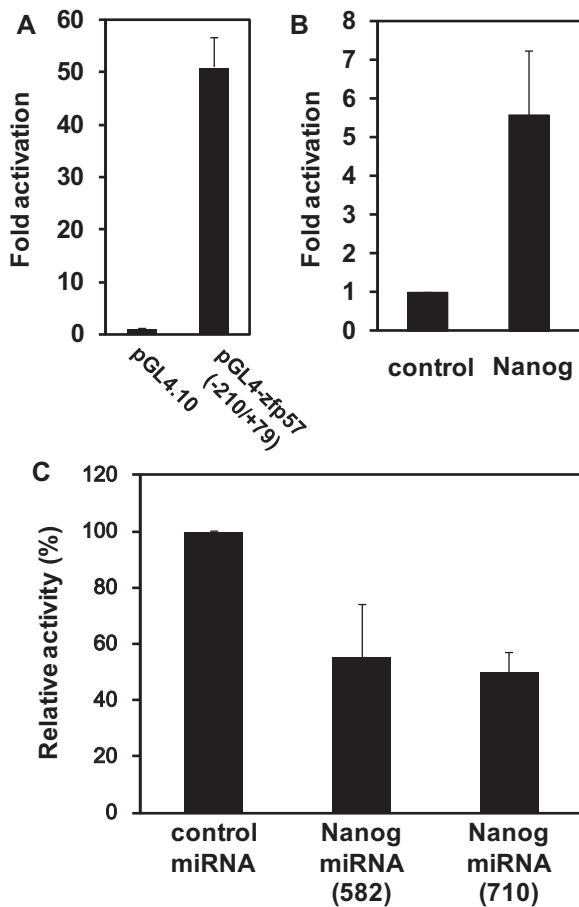


Fig. 2. Identification of a Nanog-responsive region in the *Zfp57* promoter. (A) Promoter activity of the 5'-flanking region (–210/+79) of the *Zfp57* gene in ES cells. E14 cells were transfected with pGL4.10 or pGL4-zfp57(–210/+79) and luciferase activity was examined. (B) Nanog overexpression increases the promoter activity of the *Zfp57* gene. E14 cells were transfected with pCAG-IP (control) or pCAGIP-myc-Nanog (Nanog) together with pGL4-zfp57(–210/+79) and subjected to the reporter assay. (C) Nanog knockdown decreases the promoter activity of the *Zfp57* gene. After transfection with pGL4-zfp57(–210/+79) and expression vectors for control or Nanog-targeting microRNAs, E14 cells were subjected to the reporter assay. In all panels, bars represent the mean and error bars represent the standard deviation ($n = 3$).

3.2. *Zfp57* is involved in Nanog-promoted anchorage-independent growth of ES cells

We identified *Zfp57* as a downstream molecule of Nanog; therefore, we next examined the biological role of the Nanog/*Zfp57* pathway in ES cells. We previously reported that *Zfp57* is involved in anchorage-independent growth of ES cells [15]. Nanog is involved in the anchorage-independent growth of several types of cells [21–23]. These observations raise the possibility that the Nanog/*Zfp57* axis is involved in the anchorage-independent growth of ES cells. To explore this possibility, we first investigated whether Nanog can promote anchorage-independent growth of ES cells. When Nanog was overexpressed in ES cells, the number of viable colonies in soft agar was increased, suggesting that Nanog promotes the growth of ES cells in soft agar (Fig. 3A and Supplementary Fig. S3A). On the other hand, when the expression level of *Zfp57* was reduced in Nanog-overexpressing ES cells using artificial miRNAs, the number of viable colonies decreased (Fig. 3B and Supplementary Fig. S3B and C), suggesting that *Zfp57* is involved in Nanog-promoted anchorage-independent growth of ES cells. These results suggest that the Nanog/*Zfp57* pathway controls anchorage-independent growth of ES cells.

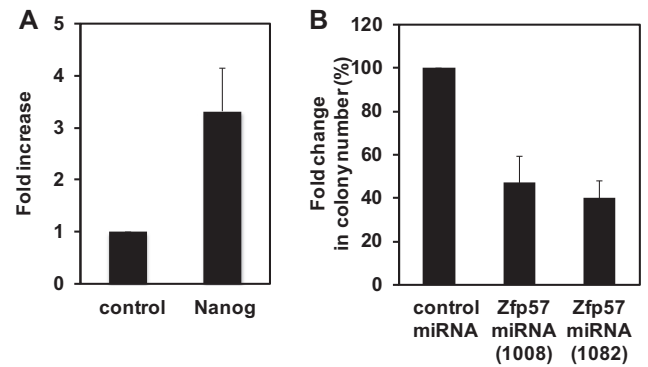


Fig. 3. Role of *Zfp57* as a downstream molecule of Nanog. (A) Nanog promotes anchorage-independent growth of ES cells. E14 cells were transfected with or without Nanog and cultured for more than 1 week in the presence of puromycin. Puromycin-resistant E14 cells were pooled and subjected to the soft agar assay. (B) *Zfp57* knockdown inhibits Nanog-enhanced anchorage-independent growth of ES cells. Nanog-overexpressing E14 cells were transfected with expression vectors for control or *Zfp57*-targeting miRNAs, cultured in the presence of hygromycin, and subjected to the soft agar assay. In both panels, bars represent the mean and error bars represent the standard deviation ($n = 3$).

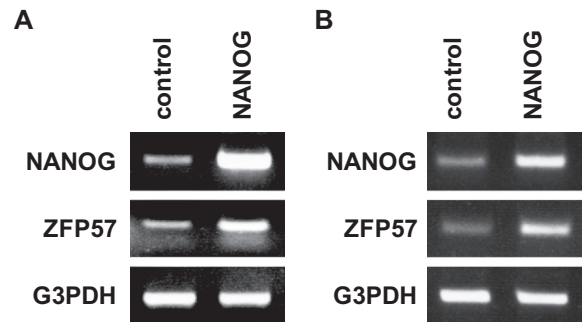


Fig. 4. NANOG overexpression increases expression of *ZFP57* in HT1080 (A) and HT29 cells (B). After transfection with or without NANOG, cells were cultured in the presence of puromycin and resistant clones were pooled and subjected to RT-PCR analysis. In both panels, data are representative of three independent experiments. G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

3.3. NANOG upregulates *ZFP57* expression in human cancer cells

To determine whether regulation of *Zfp57* expression by Nanog is restricted to ES cells, we examined the effect of NANOG overexpression on the expression level of *ZFP57* in two cancer cell lines, HT29 and HT1080, which are derived from human colorectal cancer and fibrosarcoma, respectively. In both cancer cell lines, NANOG overexpression increased the expression level of *ZFP57* (Fig. 4). These results suggest that Nanog upregulates *Zfp57* not only in mouse ES cells but also in human cancer cell lines.

4. Discussion

In this study, we demonstrated that expression of *Zfp57* is positively regulated by Nanog in ES cells. We also found that *Zfp57* is involved in Nanog-promoted anchorage-independent growth of ES cells, suggesting that the Nanog/*Zfp57* pathway plays an important role in this cell growth. In addition, we found that NANOG also regulates *ZFP57* expression in human tumor cells.

We identified *Zfp57* as a downstream molecule of Nanog. In addition, we identified a Nanog-responsive region upstream of the *Zfp57* gene. However, we do not know whether Nanog binds directly to this region to regulate *Zfp57* expression. To address this, we performed a chromatin immunoprecipitation assay; however,

we have been unable to detect the association of NANOG protein with this region thus far. It is therefore possible that Nanog regulates Zfp57 expression indirectly via another transcription factor(s).

Although we showed that the Nanog/Zfp57 pathway promotes anchorage-independent growth of ES cells, the underlying molecular mechanism remains unclear. We previously demonstrated that ZFP57 promotes the anchorage-independent growth of human fibrosarcoma cells by upregulating expression of insulin-like growth factor 2 (IGF2) [15]. However, Zfp57 knockout did not significantly reduce the expression of this growth factor in ES cells (data not shown), suggesting that Zfp57 does not upregulate IGF2 expression in these cells. Therefore, the Nanog/Zfp57 pathway may promote anchorage-independent growth of ES cells in an IGF2-independent manner.

NANOG upregulated ZFP57 expression in HT1080 cells in the current study. Additionally, we previously reported that NANOG and ZFP57 both promote anchorage-independent growth of HT1080 cells [15]. These findings suggest that NANOG promotes anchorage-independent growth of HT1080 cells via upregulating ZFP57. NANOG plays an important role in tumorigenesis [22,24] and NANOG overexpression is often associated with poor prognosis in several types of cancer [24–27]. ZFP57 is overexpressed in several types of tumor tissues [15,16]; therefore, it is possible that the NANOG/ZFP57 axis plays a central role in tumorigenesis and metastasis of several types of cancer. Further investigation of the NANOG/ZFP57 pathway would help to better understand the role of stem cell factors in oncogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.10.020>.

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