Involvement of Gli proteins in undifferentiated state maintenance and proliferation of embryonic stem cells

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Abstract

Although several important transcription factors, such as STAT3, Oct3/4, Sox2, and Nanog, have been identified for the self-renewal of mouse embryonic stem (ES) cells, the molecular mechanism behind ES cell self-renewal is not fully understood. In this study, I found that transcriptional activators Gli1 and Gli2 are highly expressed in undifferentiated ES cells and that their expression is downregulated upon differentiation. The expression of Gli1 and Gli2 was also reduced by the suppression of either Oct3/4 or Nanog, suggesting that Gli1 and Gli2 are downstream molecules of these transcription factors. Forced expression of a dominant-negative mutant of Gli2, which inhibits both Gli1 and Gli2, induced the disruption of compact colony formation, downregulation of Sox2, and upregulation of differentiation marker genes, Gata4 and Cdx2. Furthermore, the Gli2 mutant reduced the growth rate of ES cells. These results suggest that Gli1 and Gli2 are positive regulators of undifferentiated state maintenance and proliferation in ES cells.

Key words ES cells, Gli, Nanog, Oct3/4, proliferation, self-renewal

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of the mammalian blastocyst and exhibit two important characteristics: pluripotency and self-renewal [1)2)]. Self-renewal of mouse ES cells can be maintained by the presence of leukemia inhibitory factor (LIF) [3)4)]. LIF stimulation leads to the activation of the janus kinase/ signal transducer and activator of transcription 3 (STAT3) pathway, as well as the phosphoinositide 3-kinase/Akt pathway, and both pathways are integrated into a transcription factor network consisting of octamer-binding transcription factor 3/4 (Oct3/4), sex determining region Y-box2 (Sox2), and Nanog [5)6)].

Oct3/4 is involved in inner cell mass formation and is indispensable for ES cell self-renewal [7)8)]. Sox2 acts synergistically with Oct3/4 to regulate the expression of pluripotent stem cell-specific genes [9)10)]. Nanog is a homeodomain transcription factor whose overexpression prevents ES cell differentiation induced by the depletion of LIF [11)12)]. In addition to the formation of the transcription factor network, suppression of several kinases is likely to be important for ES cell self-renewal since the addition of inhibitors of extracellular signal-regulated kinase (ERK) and glycogen synthase kinase 3 (GSK3) maintains ES cell self-renewal in LIF-free medium [13)].

Despite having found the essential transcription

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Abbreviations: Cdx2, caudal type homeobox 2; Dax1, dosage-sensitive sex reversal adrenal hypoplasia critical region on chromosome X gene 1; dnGli2, dominant-negative mutant of Gli2; ERK, extracellular signal-regulated kinase; ES, embryonic stem; Fgf5, fibroblast growth factor 5; GATA4, GATA binding protein 4; Gli1/2/3, GLI-Kruppel family member GLI1/2/3; GSK3, glycogen synthase kinase 3; G3pdh, glyceraldehyde-3-phosphate dehydrogenase; LIF, leukemia inhibitory factor; Oct3/4, octamer-binding transcription factor 3/4; shRNA, short hairpin RNA; Sox2, sex determining region Y-box2; STAT3, signal transducer and activator of transcription 3; Tet, tetracycline; 2i, two inhibitors (PD0325901 and CHIR99021)

factors and repressive kinases, the detailed molecular mechanism behind ES cell self-renewal is not fully understood. Here, I report the involvement of GLI-Kruppel family member (Gli) in the maintenance of ES cells. Gli encodes a zinc-finger transcription factor that regulates the expression of its downstream genes in response to Hedgehog signaling. The Gli family consists of three isoforms, Gli1, Gli2 and Gli3, in mouse and human [14]. Gli1 is a transcriptional activator that is dependent on Gli2 and/or Gli3-mediated transcription. Gli2 is a primary mediator of Hedgehog signaling and functions primarily as a transcriptional activator, although it has a repressor function in central nervous system development. Conversely, Gli3 functions primarily as a transcriptional repressor but has an activator function in embryonic development. In this study, I found that Gli1 and Gli2 are highly expressed in undifferentiated ES cells and that the attenuation of Gli activity by dominant-negative mutant of Gli2 (dnGli2) partially perturbs the undifferentiated state of ES cells and decreases cell proliferation. These results implicate the Gli proteins in undifferentiated state maintenance and proliferation of ES cells.

Materials and Methods

Plasmid construction

Based on the Gli binding sequence reported by Sasaki et al. [15], the reporter plasmid pGL4P-8x3'Gli binding sequence was constructed by inserting 8 repeats of the following oligonucleotides into pGL4 promoter [16]: 5'-TCG ACA AGC AGG GAA CAC CCA AGT AGA AGC TC-3' and 5'-TCG AGA GCT TCT ACT TGG GTG TTC CCT GCT TG-3'. A reporter plasmid, pGL4-Gli2P500, was constructed by inserting an approximately 500 bp upstream region of the *gli2* gene (-513/+20) into pGL4.10 (Promega, Madison, USA). The coding sequence of dnGli2 was amplified from an A3-1 ES cDNA library using the following primers: 5'-GAA TTC ATG GAG ACT TCT GCC CCA GCC-3' and 5'-GCG GCC GCT AGG GGC TGG ACT GAC AAA GCC C-3'. The inducible mammalian expression vector pCAG-tTA2-IP-TRE-myc-dnGli2 was constructed by combining two gene cassettes, TRE promoter-myc-dnGli2-polyA and CAG promoter-tTA2-IRES-puromycin resistance-polyA, which are derived from pTRE-myc-dnGli2 and pCAGIP-tTA2 [17], respectively. The pTRE-myc-dnGli2 plasmid was constructed by inserting the dnGli2 cDNA into pTRE-myc (Clontech, Mountain View, USA). Construction of pSi-H1p-Nanog was described previously [17].

Cell culture

ES cell lines A3-1 [18] and ZHBTc4 [8] were cultured on gelatin-coated dishes with LIF-supplemented medium, as described previously [19]. To establish a dnGli2-inducible ES cell line (Tet-dnGli2), A3-1 cells were transfected with pCAG-tTA2-IP-TRE-myc-dnGli2 using LipofectAMINE 2000 (Invitrogen, Carlsbad, USA). Two days after transfection, cells were treated with 0.5μg/ml puromycin (Nacalai Tesque, Kyoto, Japan) for 10 days. For the two-inhibitor treatment, designed to inhibit ERK and GSK3, PD0325901 (Wako Pure Chemical, Osaka, Japan) and CHIR99021 (Wako Pure Chemical) were dissolved in dimethylsulfoxide and added into the medium at final concentrations of 1μM and 3μM, respectively.

RT-PCR Analysis

Total RNA was isolated from ES cells using Sepasol-RNA I Super G (Nacalai Tesque) and converted to cDNA using ReverTraAce (Toyobo, Osaka, Japan) with oligo(dT)$_{12-18}$ primers (Nippon EGT, Toyama, Japan). PCR products were subjected to 1.0% agarose gel electrophoresis. Primers used in this study are listed in Table 1.

Luciferase reporter assay

Using LipofectAMINE 2000 or calcium phosphate, ES cells were transfected with various plasmids and cultured for 2 or 3 days. ES cells were lysed in cell lysis buffer (20 mM Hepes-NaOH, pH 7.2, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium fluoride, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 20μg/ml aprotinin, 10μg/ml leupeptin, 10μg/ml pepstatin A, 1% Nonidet P-40, and 10% glycerol). Luciferase activity was measured using a dual luciferase assay system (Promega) and a luminometer (Luminescencer AB-2200, ATTO, Tokyo, Japan).

Western blot analysis and WST-1 assay

ES cells were harvested and lysed in the cell lysis buffer. An aliquot of lysate (50μg total protein) was subjected to Western blotting with antibodies against

Table 1. List of RT-PCR primers used for RT-PCR analysis

gene	Forward primer	Reverse primer
Cdx2	agtgattcctggggcttctt	cccttcctgatttgtggaga
Cyclin D1	caaatggaactgcttctggtg	aaagtgcgttgtgcggtag
Cyclin E1	attgtgtcctggctgaatgtc	tcgcaccactgataacctgag
Cyclin E2	tgtccagtaacagtcatctcctg	gtcatcccattccaaacctg
Dax1	cagatccgctgaactgaaca	ctacgaccgctttctccatc
Fgf5	aaagtcaatggctcccacgaa	cttcagtctgtacttcactgg
Gata4	ccgagcaggaatttgaagagg	gcctgtatgtaatgcctgcg
Gli1	tcgcagcaggaattgttgtg	tcaagagagtccagagcgtt
Gli2	ttgtcagcggatctctgaac	acatgtggatctggccatag
Gli3	gaattccaaggaggaagcggacaaagg	gaattcacctggactgtgaatggctg
G3pdh	accacagtccatgccatcac	tccaccaccctgttgctgta
Nanog	agggtctgctactgagatgctctg	caaccactggtttttctgccaccg
Oct3/4	gccctgcagaaggagctagaac	ggaatactcaatacttgatct
Patched1	cccatcgacattagtcag	gtattgctcccagaacag
Patched2	gcaccaaggaacatgctttc	ttggatgagcagcttgtagg
Sox2	atgggctctgtggtcaagtc	acccctcccaattctcttgt
T	atgccaaagaaagaaacgac	agaggctgtagaacatgatt

All primers are shown in 5' to 3' direction

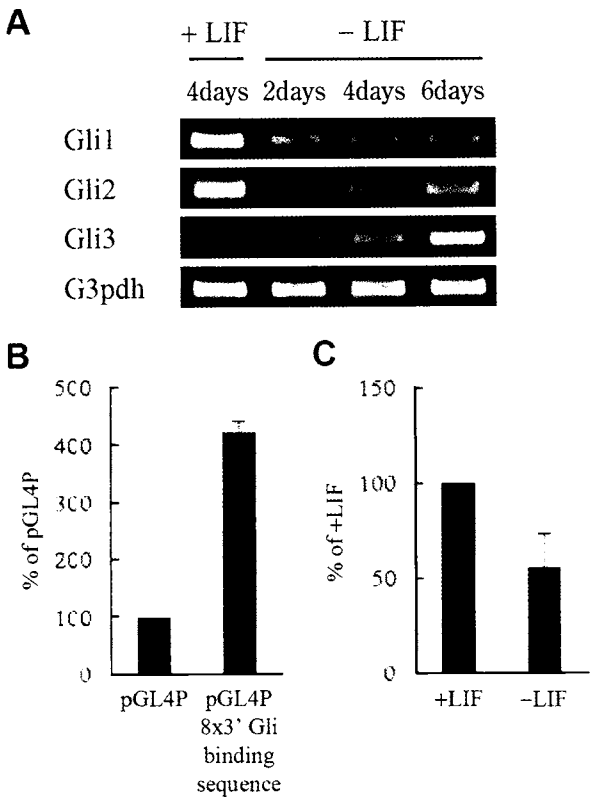

Fig. 1. Gli1 and Gli2 are expressed specifically in undifferentiated ES cells. (A) Reduction in Gli1 and Gli2 mRNA by LIF withdrawal. After culture with (+) or without (-) LIF for the indicated times, A3-1 cells were subjected to RT-PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase (G3pdh) was used as an internal control. (B) Presence of Gli activity in undifferentiated ES cells. A3-1 cells were transfected with either pGL4 promoter (pGL4P) or pGL4P-8x3'Gli binding sequence, and then subjected to luciferase assay. (C) LIF positively regulates Gli activity. One day after transfection with pGL4P-8x3'Gli binding sequence, A3-1 cells were split into two dishes and cultured for 2 days in the presence (+) or absence (-) of LIF and subjected to luciferase assay. Data shown in (A) is representative of multiple experiments. In (B) and (C), bars represent the means and standard deviations (n=3).

the Myc epitope (9E10) (Santa Cruz Biotechnology, Santa Cruz, USA), Oct3/4 (Santa Cruz Biotechnology), and α-tubulin (ICN Biomedicals, Costa Mesa, USA).

For the WST-1 assay, cells were cultured in 96-well microplates in the absence or presence of doxycycline (50ng/ml). After 3 days, cell proliferation was measured using the WST-1 cell proliferation assay (Roche, Mannheim, Germany).

Results

Regulation of Gli expression in ES cells

Preliminary microarray analysis had suggested that the transcriptional activator Gli1, as well as a stem cell-derived differentiation regulator [20], is downregulated by the removal of LIF from mouse ES cell cultures. RT-PCR analysis was conducted to verify the expression levels of Gli family genes in mouse ES cells (Fig. 1A). Gli1 and Gli2 were highly expressed in undifferentiated ES cells and downregulated during differentiation induced by LIF removal. The transcriptional activity of Gli was also detected in undifferentiated ES cells (Fig. 1B) and was reduced upon differentiation induced by LIF removal (Fig. 1C). In contrast, the expression of Gli3 was detected in differentiated ES cells but was barely detectable in undifferentiated cells (Fig. 1A), suggesting that Gli3 is not involved in ES cell self-renewal.

Nanog and Oct3/4 positively regulate Gli1 and Gli2

In general, Hedgehog signaling upregulates Gli2

activity through stabilization of Gli2 protein, and in turn, promotes transcription of Gli1. In ES cells, however, mRNA levels of both Gli1 and Gli2 were reduced during differentiation, suggesting the possibility that a self-renewal factor(s) may regulate transcription of Gli1 and Gli2. To explore this possibility, I first examined the expression levels of Gli1 and Gli2 in ZHBTc4 ES cells, where Oct3/4 expression can be suppressed by tetracycline (Tet). When Oct3/4 expression was suppressed by Tet treatment, the expression levels of both Gli1 and Gli2 decreased (Fig. 2A). Similarly, the expression of both Gli isoforms was downregulated by Nanog knockdown (Fig. 2B). Additionally, the promoter activity of an approximately 500 bp region upstream of the *gli2* gene was reduced in Oct3/4-deficient ES cells (Fig. 2C). The promoter activity of this region was also reduced when Nanog was knocked down, while it was increased by forced expression of Nanog (Fig. 2D and 2E). These results suggest that Oct3/4 and Nanog, important transcription factors for self-renewal, positively regulate the expression of Gli1 and Gli2, and raise the possibility that Gli1 and Gli2 play a role in ES cell self-renewal.

Establishment of ES cells that express dominant-negative mutant of Gli2

The role of Gli1 and Gli2 in ES cells was examined using a Gli2 mutant that lacks the C-terminal activation domain (Fig. 3A) and thereby acts as a dominant-negative inhibitor of both Gli1 and Gli2 function [21]. This mutant of Gli2 was introduced into A3-1 ES cells with a Tet-inducible system to establish Tet-dnGli2 cells. When Tet-dnGli2 cells were cultured in the absence of Tet, the dnGli2 protein was expressed (Fig. 3B) and Gli transcriptional activity was suppressed (Fig. 3C). Moreover, the expression of Gli2 downstream genes

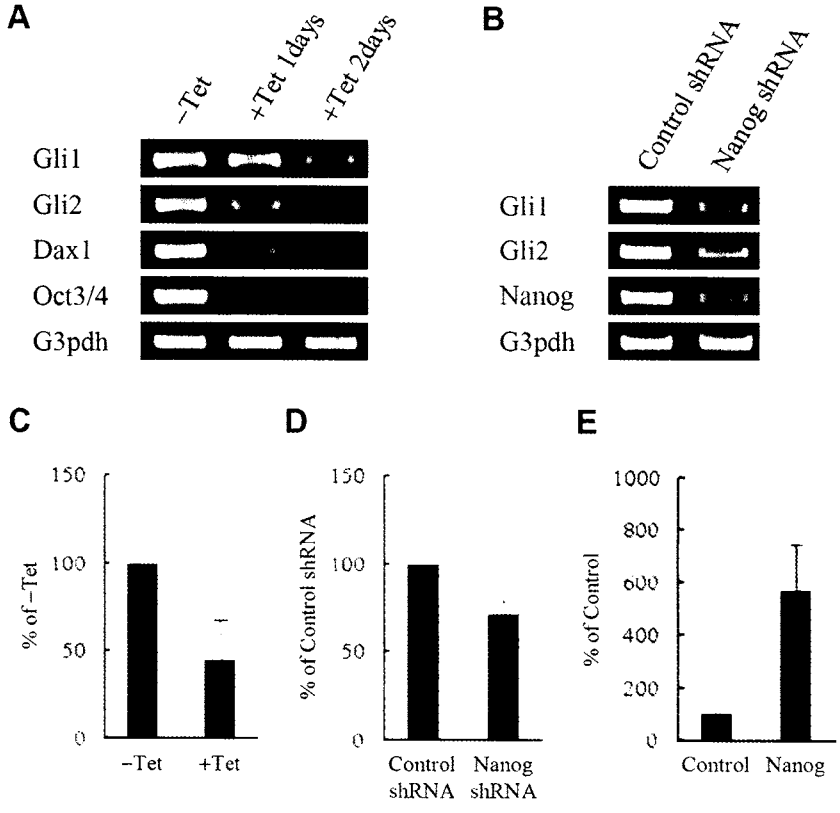

Fig. 2. Nanog and Oct3/4 positively regulate Gli1 and Gli2. (A) Gli family genes are regulated by Oct3/4. ZHBTc4 cells were cultured with (+) or without (-) Tet (1.5μg/ml) for the indicated times and subjected to RT-PCR analysis. Dosage-sensitive sex reversal adrenal hypoplasia critical region on chromosome X gene 1 (Dax1), a direct target of Oct3/4 [28], was used to confirm the downregulation of Oct3/4 activity. (B) Nanog regulates expression of Gli family genes. After transfection with pSilencer 3.1-H1 puro (control shRNA) or pSi-H1p-Nanog (Nanog shRNA), ES cells were cultured for 3 days in the presence of puromycin and subjected to RT-PCR analysis. (C) Gli promoter activity is regulated by Oct3/4. ZHBTc4 cells were transfected with pGL4-Gli2P500 and then cultured for 2 days in the presence (+) or absence (-) of Tet. (D) Gli promoter activity is downregulated by Nanog knockdown. pGL4-Gli2P500 was co-transfected into A3-1 cells with pSilencer 3.1-H1 puro (control shRNA) or pSi-H1p-Nanog (Nanog shRNA) and cultured for 2 days. (E) Nanog overexpression increases Gli promoter activity. pGL4-Gli2P500 was co-transfected into A3-1 cells with pCAGIPmyc (control) or pCAGIPmyc-Nanog (Nanog) and cultured for 2 days. Data shown in (A) and (B) are representative of multiple experiments. Bars represent the means and standard deviations (n=3 or more).

including Gli1, Patched1 and Patched2, was downregulated by ectopic expression of dnGli2 (Fig. 3D). These results indicate that Tet-dnGli2 cells are useful for examining the role of Gli1 and Gli2 in ES cells.

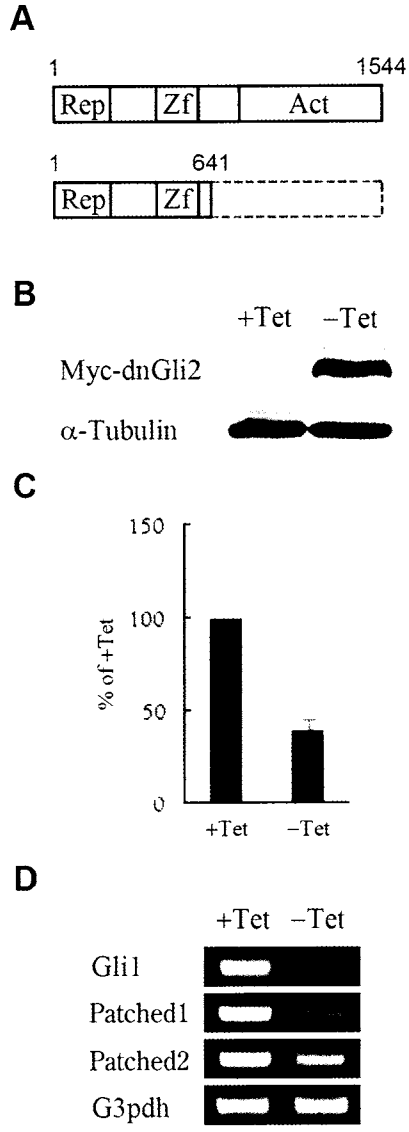

Fig. 3. Establishment of the Tet-dnGli2 cell line using a Tet-inducible expression system. (A) Diagram of full-length Gli2 and dnGli2. The dnGli2 lacks the transcriptional activation domain. Rep, repressor domain. Zf, zinc-finger domain. Act, activation domain. (B) Expression of myc-tagged dnGli2 protein is strictly regulated by Tet. After culture with (+) or without (-) Tet for 3 days, Tet-dnGli2 cells were subjected to Western blot analysis using an antibody against the Myc-tag. α-Tubulin was used as a loading control. (C) DnGli2 represses Gli transcriptional activity. One day after transfection with pGL4P-8x3'Gli binding sequence, Tet-dnGli2 cells were split into two dishes and cultured for 1 day in the presence (+) or absence (-) of Tet. Bars represent the means and standard deviations (n=3). (D) Downregulation of Gli2 target genes upon Tet withdrawal. After culture in the presence (+) or absence (-) of Tet for 3 days, Tet-dnGli2 cells were subjected to RT-PCR analysis. In (B) and (D), data are representative of multiple experiments.

Effect of dnGli2 expression on ES cell self-renewal

A comparison of the morphology of Tet-dnGli2 cells in the presence or absence of Tet showed that dnGli2-expressing ES cells failed to form compact colonies, a characteristic feature of undifferentiated ES cells (Fig. 4A). Furthermore, an important self-renewal factor, Sox2, was downregulated, and expression of an endodermal marker GATA binding protein 4 (Gata4) and a trophectodermal marker

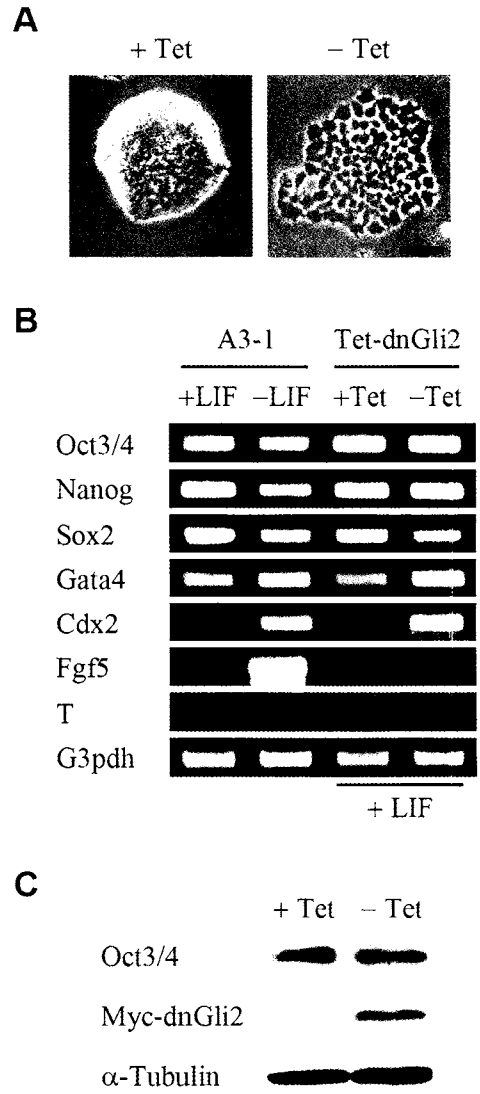

Fig. 4. Gli plays a positive role in maintenance of undifferentiated state. (A) Morphological changes in Tet-dnGli2 cells. Tet-dnGli2 cells were cultured with (+) or without (-) Tet for 3 days in the presence of LIF. Bars, 50 μm. (B) Gene expression in Tet-dnGli2 cells. A3-1 cells were cultured in the presence (+) or absence (-) of LIF for 3 days. Tet-dnGli2 cells were cultured in the presence of LIF with (+) or without (-) Tet for 3 days. In both experiments, cells were harvested and subjected to RT-PCR analysis. (C) DnGli2 shows no effect on expression levels of Oct3/4 protein. Tet-dnGli2 cells were cultured with (+) or without (-) Tet for 3 days and subjected to Western blot analysis using antibodies against Oct3/4, Myc-tag and α-tubulin. Results are representative of multiple experiments.

caudal type homeobox 2 (Cdx2) was induced in dnGli2-expressing cells (Fig. 4B). However, the expression of dnGli2 showed no effect on the expression levels of the self-renewal markers Oct3/4 and Nanog, a mesodermal marker T, and an ectodermal marker fibroblast growth factor 5 (Fgf5) (Fig. 4B and 4C). The dnGli2-expressing ES cells could be maintained for 3 weeks (data not shown). These results suggest that Gli is involved in repressing ES cell differentiation, although the factor is dispensable for self-renewal.

Two-inhibitor treatment did not suppress the abnormal phenotype of dnGli2-expressing ES cells

Recent reports have suggested that the addition of two inhibitors (2i), one against ERK and the other against GSK3, rescued the abnormal phenotype of ES mutant cells [13)22)]. Therefore, the two-inhibitor treatment was used to rescue the phenotype of dnGli2-expressing ES cells. Although the two-inhibitor treatment inhibited ES cell differentiation by LIF removal, these inhibitors did not show any effect on cellular morphology or gene expression in dnGli2-expressing cells (Fig. 5A and 5B). These results suggest that two-inhibitor treatment fails to rescue the abnormal phenotype of dnGli2-expressing ES cells.

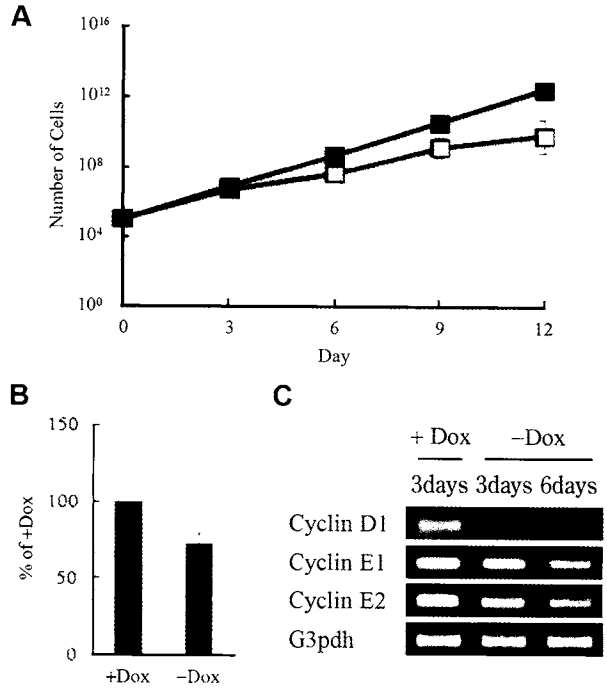

Fig. 6. Gli plays an important role in the proliferation of ES cells. (A) Proliferation curve of Tet-dnGli2 cells. In the presence of LIF, Tet-dnGli2 cells were cultured with (black square) or without (white square) doxycycline (Dox) for the indicated days. (B) Tet-dnGli2 cells were cultured in the presence of LIF with (+) or without (-) Dox for 3 days, and then subjected to a WST-1 cell proliferation assay. Bars represent the means and standard deviations (n=3). (C) Expression of cell cycle-related genes in Tet-dnGli2 cells. Tet-dnGli2 cells were cultured in the presence of LIF with (+) or without (-) Dox for the indicated periods and subjected to RT-PCR analysis. Results are representative of multiple experiments.

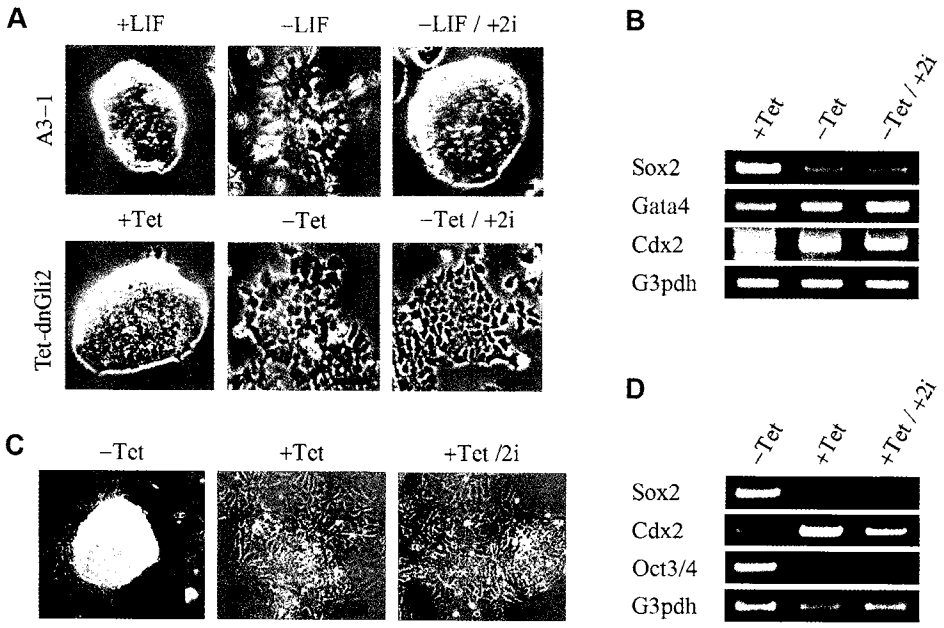

Fig. 5. Two-inhibitor treatment failed to rescue the dnGli2-expressing ES cells phenotype. Co-treatment with ERK inhibitor and GSK3 inhibitor does not restore the normal morphology (A) or gene expression (B) in dnGli2-expressing ES cells. Tet-dnGli2 cells were cultured in the presence (+) or absence (-) of Tet with or without two inhibitors (2i) for 3 days. As a control, A3-1 cells were cultured in the presence (+) or absence (-) of LIF with or without 2i. The two-inhibitor treatment did not prevent changes in morphology (C) or gene expression (D) in Oct3/4-null ES cells. ZHBTc4 cells were cultured in the presence (+) or absence (-) of Tet with or without 2i for 3 days. Data shown are representative of multiple experiments. Bars, 50 μm.

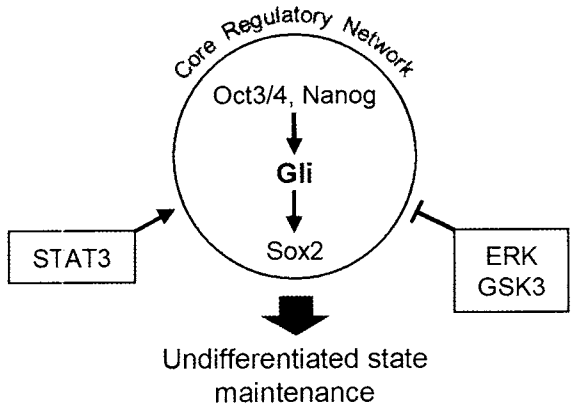

Fig. 7. Proposed role of Gli in ES cell self-renewal. Gli may act as a mediator of core regulatory network to contribute to maintenance of undifferentiated state.

In addition, I examined the effect of 2i on differentiation induced by Oct3/4 deficiency since Gli is downstream gene of Oct3/4. As shown in Fig. 5C and 5D, Tet-treated ZHBTc4 ES cells differentiated into trophectodermal cells with induction of Cdx2 even in the presence of 2i, suggesting that 2i cannot inhibit the differentiation of Oct3/4-null ES cells.

Effect of dnGli2 expression on ES cell growth

In *Drosophila*, Hedgehog signaling promotes cell growth through upregulation of cyclin D and cyclin E [23]. I therefore examined the effect of dnGli2 on cellular growth of ES cells. When Tet-dnGli2 cells were cultured with or without doxycycline, which mimics Tet, the growth rate of Tet-dnGli2 cells was reduced in the absence of doxycycline, as determined by cell counts (Fig. 6A). Similar results were obtained using a WST-1 cell proliferation colorimetric assay (Fig. 6B). Consistent with these results, the cell cycle-related genes cyclin D1, cyclin E1 and cyclin E2 were downregulated by dnGli2 (Fig. 6C). These results suggest that Gli is involved in ES cell growth.

Discussion

Hedgehog signaling is highly conserved from insects to vertebrates. In the presence of the Hedgehog ligand, the full-length form of Gli2 is transported to the nucleus and positively regulates the expression of target genes including Gli1. In the absence of Hedgehog, on the other hand, the C-terminal portion of Gli2 is cleaved off and the truncated Gli2 acts as a repressor. Although the Hedgehog signaling pathway plays a pivotal role in organogenesis and differentiation during development, the role of Hedgehog signaling in ES cells is not clearly understood. In this study, using a dnGli2, I provide direct evidence that Gli plays a role in the undifferentiated state maintenance and proliferation of mouse ES cells (Fig. 7).

In ES cells, both Gli1 and Gli2 transcripts were downregulated simultaneously during differentiation. In Hedgehog signaling, Gli1 is a direct target of Gli2. It is likely, therefore, that the downregulation of Gli1 is due to the downregulation of Gli2. However, Gli2 activity is usually regulated at the post-translational level through protein cleavage. Interestingly, I found that Gli2 activity is regulated at the transcriptional level in self-renewing ES cells: the level of Gli2 mRNA decreases upon ES cell differentiation (Fig. 1A). Moreover, I found that the artificial downregulation of Oct3/4 or Nanog reduces the mRNA level of Gli2 (Fig. 2A and 2B), suggesting that Gli2 is a common downstream target of these two factors. Supporting these results, I found that an approximately 500 bp region upstream of the *gli2* gene responds to Nanog and Oct3/4 (Fig. 2C and 2D and 2E). These observations suggest that the downregulation of Gli2 upon LIF removal may be due to a summed effect of simultaneous downregulation of Oct3/4 and Nanog during ES cell differentiation.

Ectopic expression of dnGli2 in ES cells led to the disruption of compact colony formation, downregulation of Sox2 and upregulation of Gata4 and Cdx2 (Fig. 4A and 4B), suggesting that Gli promotes the maintenance of undifferentiated state in ES cells. The molecular mechanism by which the suppression of Gli activity interferes with the undifferentiated state of ES cells is unknown. Since an important transcription factor, Sox2, is downregulated by Gli suppression, it is possible that the downregulation of Sox2 explains, at least in part, the phenotype of dnGli2-expressing ES cells. This possibility is supported by a study using Sox2-knockout ES cells that showed that Sox2 is involved in the repression of Gata4 [10]. However, the same study also showed that Sox2 does not participate in the repression of Cdx2. It is therefore unlikely that the downregulation of Sox2 is solely responsible for the abnormal phenotype of dnGli2-expressing cells. It has been shown that Cdx2 forms a reciprocal inhibitory loop with Oct3/4 in ES cells [24]. In the

present study, I found that, although dnGli2 induced the expression of Cdx2, the expression level of Oct3/4 was normal in dnGli2-expressing cells (Fig. 4B and 4C). These results suggest that the observed upregulation of Cdx2 was not enough to have an inhibitory effect on Oct3/4 in the presence of LIF. This may explain why dnGli2-expressing ES cells can maintain their self-renewal.

The aberrant phenotype of dnGli2-expressing ES cells could not be rescued by the two-inhibitor treatment (Fig. 5A and 5B). In contrast, the phenotype of STAT3- and myc-associated factor X-null ES cells was rescued by the combined inhibition of GSK3 and ERK [13)22)]. Extensive studies have revealed that ES cell self-renewal is controlled by a pluripotent gene regulatory network that includes Oct3/4 [6)25)26)]. STAT3 positively regulates this network, while ERK and GSK3 have negative effects on the network. Therefore, the two-inhibitor treatment has been proposed to maintain ES cell self-renewal by canceling the ERK- and GSK3-mediated repressive effects on the regulatory network [27)]. If this is the case, the two-inhibitor treatment should not rescue the phenotype caused by the deficiency of a transcription factor that participates in the pluripotent gene regulatory network. Indeed, I observed that the two-inhibitor treatment failed to rescue the differentiation of Oct3/4-null ES cells (Fig. 5C and 5D). Thus, my present observation that the two-inhibitor treatment failed to rescue the phenotype of dnGli2-expressing ES cells suggests the possibility that Gli may be a component of the core regulatory network (Fig. 7). Further analysis of roles of Gli family proteins in ES cells may contribute to understand the molecular mechanism of ES cell regulation.

Acknowledgments

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