ARTICLE
Journal of Cellular Biochemistry 106:1041–1047 (2009)

Journal of Cellular Biochemistry

Exogenous Nanog Alleviates But Is Insufficient to Reverse Embryonic Stem Cells Differentiation Induced by PI3K Signaling Inhibition

Na Liu,[1,2] Min Lu,[1] Xiao Ming Feng,[1] Feng Xia Ma,[1] Zhi Hong Fang,[1] Xue Mei Tian,[1] Qian Ren,[1] Lei Zhang,[1] Bin Liu,[1] Ping Ping Huang,[1] Lin Liu,[2] and Zhong Chao Han[1*]

[1] *State Key Laboratory of Experimental Hematology, Institute of Hematology, National Research Center for Stem Cell Engineering and Technology, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China*

[2] *College of Life Science, Nankai University, Tianjin, China*

ABSTRACT

PI3K signaling pathway plays a significant role in embryonic stem cells (ES cells) self-renewal. Overexpression of Nanog maintains mouse ES cells pluripotency independent of leukemia inhibitory factor (LIF). However, little is known about the effect of PI3K signaling pathway on ES cells with Nanog overexpression. Our experiments aimed to explore the relationship between PI3K signaling pathway and Nanog expression in ES cells. We observed the effect of LY294002, a specific inhibitor of PI3K pathway, on wild-type J1 cells and Nanog overexpressing (Ex-Nanog) J1 cells in the presence or absence of LIF. With LY294002 treatment, both of them lost their ES features even in the presence of LIF. But the differentiation induced by LY294002 on Ex-Nanog J1 cells was slighter lower than that on wild-type J1 cells. These results indicate that inhibition of PI3K pathway induces mouse ES cells differentiation. Exogenous Nanog sustains mouse ES cells pluripotency independent of LIF, and alleviates the differentiation induced by LY294002. But it is insufficient to totally reverse the differentiation. J. Cell. Biochem. 106: 1041–1047, 2009.

KEY WORDS: EMBRYONIC STEM CELLS; NANOG; PI3K SIGNALING PATHWAY; PLURIPOTENCY

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocyst and possess pluripotency [Evans and Kaufman, 1981; Thomson et al., 1998]. ES cells can be expanded continuously when co-cultured with mitotic inactivated embryonic fibroblast cells (MEF) or leukemia inhibitory factor (LIF) [Evans and Kaufman, 1981; Smith et al., 1988]. LIF sustains mouse ES cells self-renewal through STAT3 [Niwa et al., 1998], whose activation is sufficient to prevent mouse ES cells differentiation [Matsuda et al., 1999]. Furthermore, bone morphogenetic protein 4 (BMP4) cooperates with LIF to maintain the pluripotency of mouse ES cells [Ying et al., 2003]. However, LIF-STAT3 is not sufficient to maintain human ES cells pluripotency [Reubinoff et al., 2000; Dahéron et al., 2004]. It has been found that maintenance of pluripotency of human ES cells is independent of LIF-STAT3 signaling pathway [Humphrey et al., 2004] and that basic fibroblast growth factor (bFGF) sustains undifferentiated state of human ES cells in the presence of Noggin, an inhibitor of BMP signaling [Xu et al., 2005]. Other evidence suggests that the ERK [Hamazaki et al., 2006; Li et al., 2007], Wnt [Sato et al., 2004; Liu et al., 2007; Miyabayashi et al., 2007] signaling pathway may be necessary for maintenance of pluripotency of both human and mouse ES cells. Recently, several studies have indicated that phosphoinositide 3-kinase (PI3K) pathway is required for ES cells proliferation [Jirmanova et al., 2002; Hallmann et al., 2003; Pyle et al., 2006]. Moreover, PI3K pathway may be crucial for ES cells self-renewal [Takahashi et al., 2005]. Inhibition of PI3K signaling pathway in mouse and human ES cells has been reported to induce differentiation in the presence or absence of LIF [Paling et al., 2004; Armstrong et al., 2006]. Activation of Akt signaling is sufficient to maintain pluripotency of mouse and primate ES cells [Watanabe et al., 2006], suggesting that PI3K/Akt signaling is necessary for the maintenance of ES cells pluripotency.

Nanog is a homeobox-containing transcription factor with a critical role in maintaining the pluripotency of ICM and ES cells. Nanog maintain ES cells undifferentiated state by impressing

Grant sponsor: Ministry Science & Technology of China; Grant number: 2006AA02A110; Grant sponsor: National Natural Science Foundation of China; Grant numbers: 30570357, 30600238; Grant sponsor: Tianjin Municipal Science and Technology Commission; Grant numbers: 06YFSZSF01300, 07JCYBJC11200.

*Correspondence to: Prof. Zhong Chao Han, Institute of Hematology, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300020, China. E-mail: tihzchan@public.tpt.tj.cn

Received 10 October 2008; Accepted 7 January 2009 • DOI 10.1002/jcb.22082 • 2009 Wiley-Liss, Inc.
Published online 19 February 2009 in Wiley InterScience (www.interscience.wiley.com).

differentiate-related genes expression and increasing pluripotency genes level [Liu et al., 2008]. Disruption of Nanog in ES cells results in differentiation to extra-embryonic endoderm lineages. Over-expression of Nanog renders ES cells self-renewal independent of LIF, STAT3, and BMP4 [Chambers et al., 2003; Mitsui et al., 2003; Hyslop et al., 2005]. Here, we aimed to investigate the relationship between exogenous Nanog and PI3K signaling pathway on mouse ES cell pluripotency. Our experimental results demonstrate that exogenous Nanog can maintain mouse ES cells pluripotency independent of LIF and alleviate LY294002-induced differentiation but cannot completely reverse cell differentiation.

MATERIALS AND METHODS

CELL CULTURE

J1 mouse ES cells (kindly provided by Dr. Zhengyu Wang (Harvard University)), were cultured as previously reported by our laboratory [Li et al., 2005]. The medium consisted of high glucose Dulbecco's modified eagle medium (DMEM) (no-pyruvate, high-glucose formulation; GIBCO-BRL) supplemented with 15% fetal bovine serum (Hyclone), 1× nonessential amino acids (Hyclone), 2.0 mM L-glutamine, 1,000 U/ml mouse recombine LIF (Chemicon), 100 μM 2-mercaptoethanol (Sigma), 100 U penicillin, and 100 μg/ml streptomycin. 3T3 cells were cultured in high-glucose DMEM supplemented with 15% fetal bovine serum (Hyclone), 2.0 mM L-glutamine, 100 U penicillin, and 100 μg/ml streptomycin.

PI3K signaling pathway inhibitor LY294002 was purchased from Sigma. The final concentration at 10 μM was used. Solvent DMSO served as negative control.

RT-PCR

Total RNA was extracted with Trizol (Invitrogen). Two micrograms of RNA was reversely transcribed into cDNA using MMLV reverse transcriptase (Promega) in a volume of 50 μl. The following primers were used: GFP: forward 5′-cagaagaacggcatcaaggtg and reverse 5′-cggactgggtgctcaggtag; GAPDH: forward 5′-cacttgaagggtggagc and reverse 5′-gggctaagcagttggtg.

PLASMID CONSTRUCTION

Mouse Nanog cDNA was PCR-amplified by LA-Taq polymerase (TAKARA Bio, Japan) using mRNA derived from wild-type J1 cells and primer sequences, which contained *Bgl*II and *Sal*I restriction sites, then subcloned into pEGFP–N1 (Clontech) between *Bgl*II and *Sal*I sites. In order to get Nanog–GFP fusion protein, the termination code of Nanog was deleted. Empty pEGFP–N1 served as a negative control.

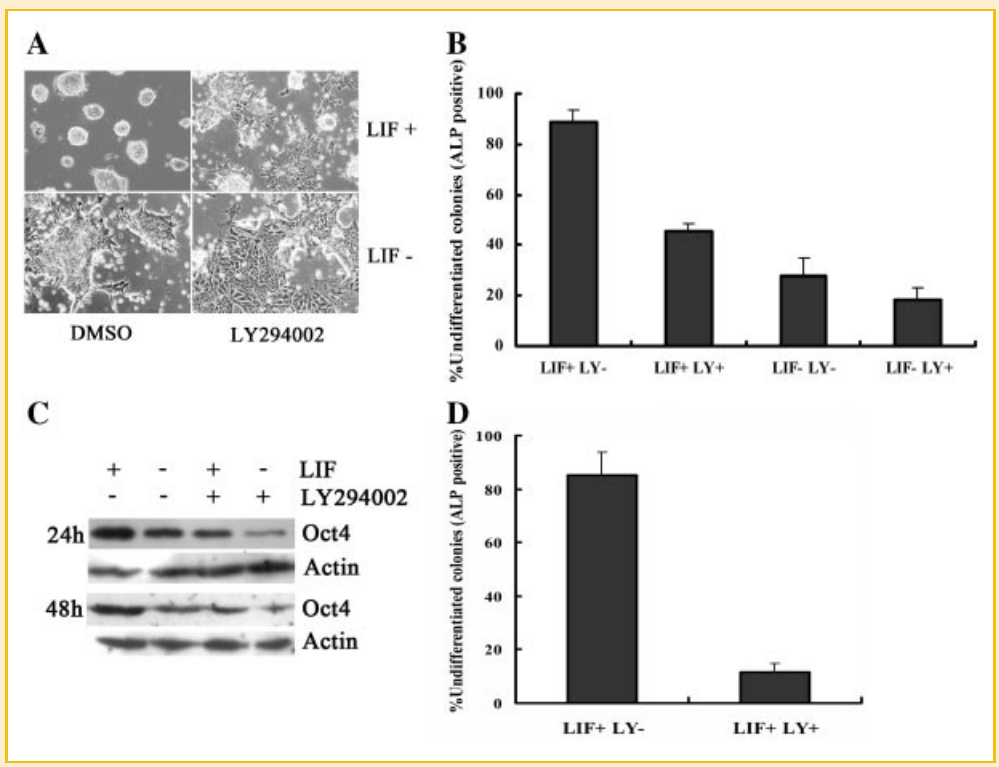

Fig. 1. LY294002 induces differentiation of ES cells. A: J1 cells were cultured for 3 days in the presence or absence of 1,000 U/ml LIF and 10μM LY294002. B: Quantitative analysis of ALP positive colonies 3 days after LY294002 treatment. Triplicate counts were performed, and the percentage of ALP positive colonies was determined for each treatment. Data represents Mean ± SD. C: Oct4 protein expression was detected by Western blot. Actin was used to verify equal loading. D: Quantitative analysis of ALP positive colonies 7 days after LY294002 treatment. Triplicate counts were performed, and the percentage of ALP positive colonies was determined for each treatment. Data represents Mean ± SD.

TRANSFECTION

Transfection was carried out with LipofectAMINE 2000 (Invitrogen) following the manufacturer's instruction. Nanog-overexpressing (Ex-Nanog) J1 cells were established by G418 (400 μg/ml, Sigma) selection for 2 weeks following transfection. Mixed population was selected and used for our further investigation.

ALKALINE PHOSPHATASE STAINING

Alkaline phosphatase (ALP) expression level was detected by staining with a diagnostic kit, ALP substrate kit (product of our institute), according to the instruction.

FLOW CYTOMETRY ANALYSIS

ES cells were washed with PBS (including 5% BSA), and then incubated with PE-conjugated anti-SSEA1 mouse monoclonal antibody (Santa Cruz Biotechnology) at 4 for 30 min. Then cells were washed three times. FACS analysis was performed using a FACScan Flow Cytometer (BD Biosciences).

WESTERN BLOTTING

Cells were collected and washed twice with cold phosphate-buffered saline (PBS). Then cells were resuspended in cell lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na4P2O7, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.25% deoxycholate, and 0.1% SDS. Lysates were electrophoresed using SDS–PAGE (10% polyacrylamide gel) and blotted 1.5 h onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked overnight with 5% fat-free milk solution. Samples were probed with antibody anti-Nanog 1/1,000 (BETHYL), anti-Oct4 1/200 (Santa Cruz Biotechnology) and anti-Actin 1/200 (Santa Cruz Biotechnology). Anti-goat or anti-mouse IgG-peroxidase conjugate (Sigma) were diluted at 1:5,000. Staining was visualized using an electogenerated chemiluminescence (ECL) kit (Amersham Biosciences Corp.).

RESULTS

LY294002 INDUCES DIFFERENTIATION OF ES CELLS

It has been reported that PI3K signaling pathway plays an important role in ES cells self-renewal. Here, we examined the role of LY294002 in ES cells pluripotency, which is a specific inhibitor of PI3K pathway [Vlahos et al., 1994]. With the treatment of LY294002 (10 μM) for 3 days, ES cells displayed differentiated phenotype, lost the compact colony feature and became flat even in the presence of LIF (Fig. 1A). As the ALP and Oct4 are well-known markers of pluripotency in ES cells, the cell pluripotency state was then quantified by ALP activity. Cultivation of J1 cells in the presence of LIF maintained ALP positive clones at about 90%. When J1 cells were treated with LY294002 in the absence of LIF for 3 days, approximately 80% of the cells differentiated and lost ALP expression. The presence of LIF inhibited the differentiation caused by LY294002. About 45% cells formed ALP positive clones in the presence of LIF and LY294002 (Fig. 1B). We then analyzed Oct4 expression level and the results obtained confirmed that obtained by ALP analysis. Oct4 was highly expressed in J1 cells cultured with LIF. After LY294002 treatment for 48 h, Oct4 expression was decreased significantly (Fig. 1C). With the treatment of LY294002 for 7 day, only 10% cells were ALP positive (Fig. 1D).

LY294002 REDUCES NANOG EXPRESSION IN MOUSE ES CELLS

To investigate the effect of PI3K pathway on ES cells pluripotency in detail, we then analyzed Nanog expression in the presence or absence of LY294002. J1 cells were cultured in the presence of LIF and LY294002 alone or in combination. Four hours after LY294002 treatment, Nanog expression was instantly decreased. Twenty-four hours after LY294002 treatment, Nanog expression was substantially decreased, becoming barely detectable at 48 h in the presence of LY294002 alone. LIF significantly reduced the effect of LY294002 on Nanog downregulation (Fig. 2).

In our experiment, we observed that Nanog expression was reduced as soon as LY294002 was added, which suggested that PI3K pathway probably regulated Nanog expression in ES cells [Storm et al., 2007]. To explore the relationship between Nanog expression and PI3K signaling pathway, we next examined whether forced expression of exogenous Nanog was able to prevent LY294002-induced differentiation in ES cells.

FORCED EXOGENOUS NANOG EXPRESSION MAINTAINS THE PLURIPOTENCY OF ES CELLS INDEPENDENT OF LIF

To explore the effect of exogenous Nanog on mouse ES cells differentiation induced by PI3K pathway inhibition, we firstly forced exogenous Nanog expression in J1 cells. The molecular weight of exogenous Nanog was larger than endogenous Nanog, because it was a fusion protein of Nanog and GFP, which rendered us to distinguish

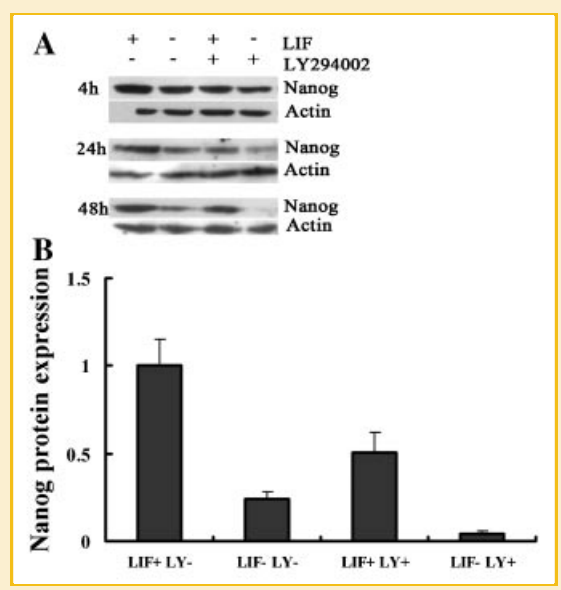

Fig. 2. LY294002 decreases Nanog expression in mouse ES cells. A: Nanog expression was analyzed by immunoblotting. J1 cells were incubated for 4, 24, 48 h with LIF and LY294002 alone or LIF plus LY294002. Actin was used to verify equal loading. B: The precise Nanog expression levels at 48 h were quantified by densitometry with Bandscan software. Nanog expression was normalized by Actin expression. A value of 1 was set to J1 cells cultured with LIF (LIF+ LY−). Data represents as Mean ± SD of three independent experiments.

it from endogenous Nanog. In pEGFP–N1 J1 cells (with empty vector), only endogenous Nanog protein was detected. Two protein strips were seen in Ex-Nanog J1 cells. One was endogenous Nanog protein; another was exogenous Nanog–GFP fusion protein with a larger molecular mass than endogenous Nanog (Fig. 3A).

Our next goal was to verify whether Nanog–GFP fusion protein was functional in ES cells pluripotency. In our experiment, we observed that J1 cells lost their ES cells feature in the absence of LIF. When J1 cells were cultured in the absence of LIF for 6 days (three passages), the number of ALP positive clones was decreased to less than 10% (Fig. 3B,E). J1 cells with exogenous Nanog (Ex-Nanog J1) maintained ES cells morphology in the absence of LIF. Ex-Nanog J1 cells were able to be passaged at least 15 passages and maintained the undifferentiated state in the absence of LIF. Ex-Nanog J1 cells grew in compact colonies (Fig. 3D). ALP colony assay was then performed to determine cell morphology and differentiation. Wild-type J1 cells showed reduced number of ALP positive colonies at the third passage (about 10%). Instead, when Ex-Nanog J1 cells were passaged for 1 month (15 passages) in the absence of LIF, about 85% of the colonies were ALP positive, which was similar to cells cultured with LIF (Fig. 3C,E). These results also indicated Nanog–GFP fusion protein was fully functional.

EXOGENOUS NANOG ALLEVIATES LY294002-INDUCED DIFFERENTIATION

Our results showed that PI3K pathway inhibition induced ES cells differentiation (Fig. 1). Exogenous Nanog was able to sustain mouse ES cells pluripotency in the absence of LIF (Fig. 3). It was therefore interesting to investigate the effect of PI3K pathway cooperated with exogenous Nanog on mouse ES cells pluripotency.

Wild-type J1 cells or Ex-Nanog J1 cells were treated with LIF and LY294002 together or each alone for 48 h. Wild-type J1 cells displayed differentiated phenotype after treatment of LIF and LY294002 for 48 h (LIF+ LY+). Some cells lost the compact colony feature and became flat (Fig. 4Ab). ALP positive rate was also reduced. Similar results were observed in Ex-Nanog J1 cells (Fig. 4Bb). But the differentiated degree in Ex-Nanog J1 cells was slighter lower than that in wild-type J1 cells. In the absence of LIF, significant differentiation was observed in wild-type J1 cells treated with LY294002 (LIF− LY+) or not (LIF− LY−) (Fig. 4Ad,c). But Ex-Nanog J1 cells maintained ES cells morphology and displayed undifferentiated phenotype in the absence of LIF and LY294002 (LIF− LY−) (Fig. 4Bc). With addition of LY294002 (LIF− LY+), Ex-Nanog J1 cells also lost ES cells feature. ALP activity gradually decreased (Fig. 4Bd).

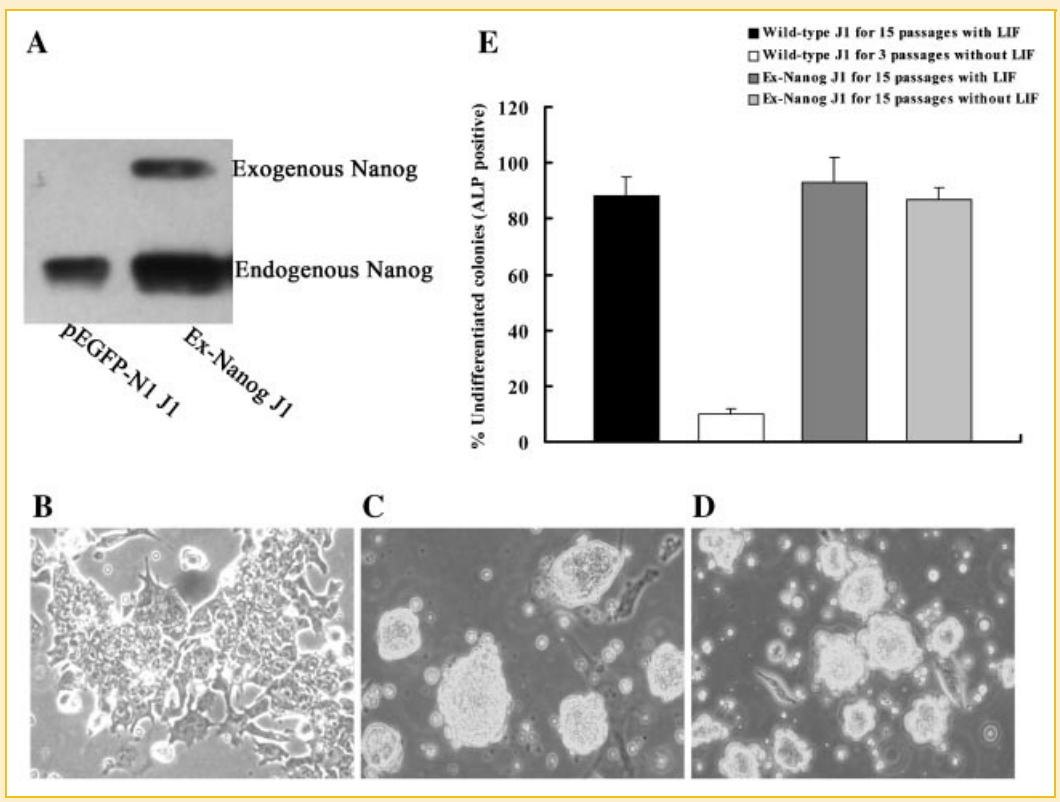

Fig. 3. Forced exogenous Nanog expression maintains pluripotency of ES cells independent of LIF. A: Western blot analyzed the expression of Nanog–GFP fusion protein in J1 cells. In pEGFP–N1 J1 cells (with empty vector), only endogenous Nanog protein was detected. Two protein strips were seen in Ex-Nanog J1 cells. One was endogenous Nanog protein; another was exogenous Nanog–GFP fusion protein with a larger molecular mass than endogenous Nanog. B: Morphology of Wild-type J1 cells cultured in the absence of LIF for 6 days (three passages). C,D: Morphology of Ex-Nanog J1 cells cultured in the presence of LIF (C) or absence (D) for 30 days (15 passages). E: The percentage of ALP positive colonies was determined for each treatment. Data represents as Mean ± SD of three independent experiments.

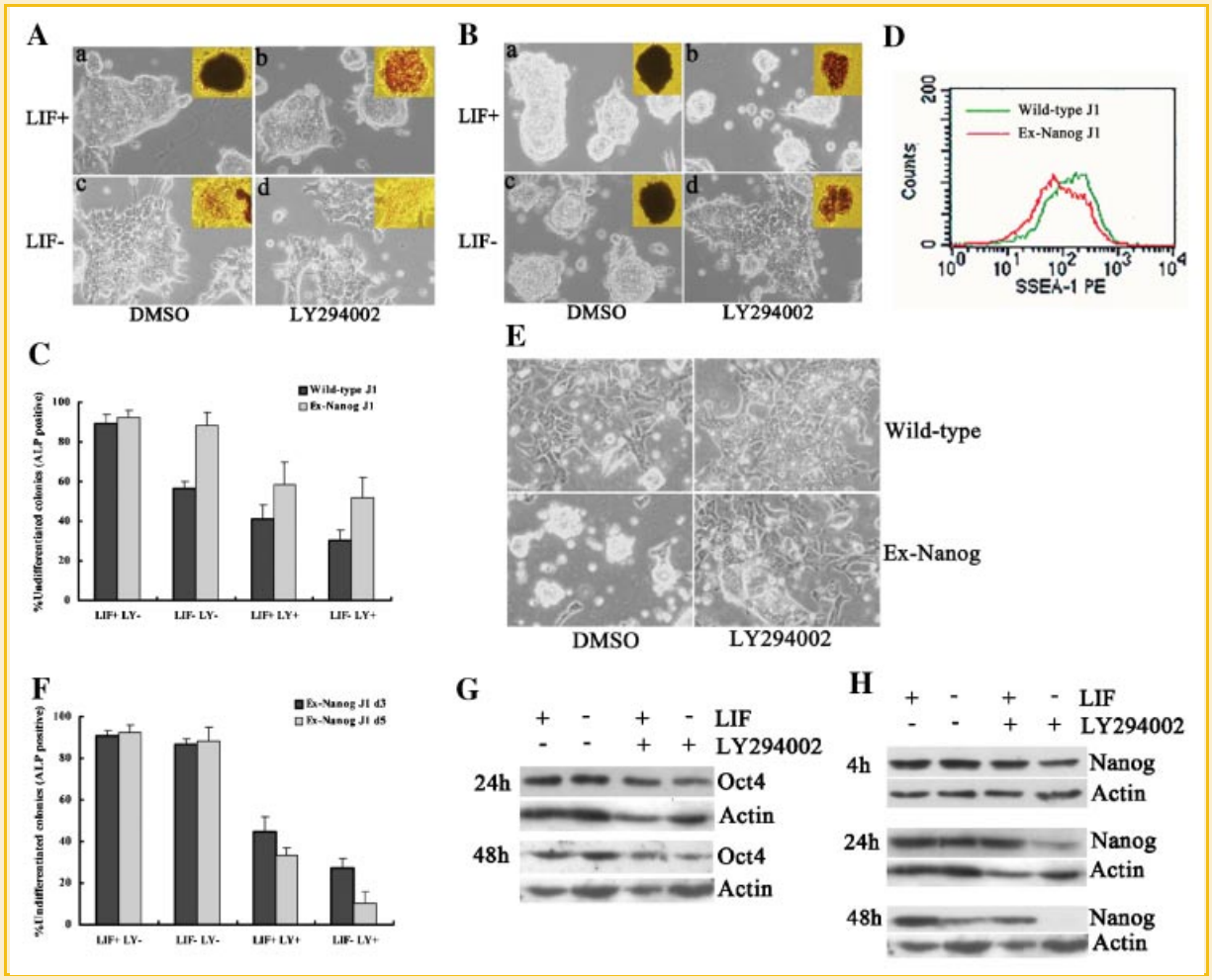

Fig. 4. Exogenous Nanog alleviates but is insufficient to reverse LY294002-induced differentiation. A,B: Photographs of wild-type J1 cells (A) and Ex-Nanog J1 cells (B) with the treatment of LY294002 for 48 h. C: Wild-type J1 cells or Ex-Nanog J1 cells were cultured in the presence of LIF or LY294002 alone or both for 48 h, and cells were stained for ALP activity using ALP diagnostic kit. Data represents as Mean ± SD of three independent experiments. D: SSEA-1 expression was analyzed by Flow Cytometry. Wild-type J1 cells and Ex-Nanog J1 cells were treated with LY294002 for 48 h in the presence of LIF. Data was representative of three independent experiments. E: Photographs of wild-type J1 cells and Ex-Nanog J1 cells with the treatment of LY294002 for 5 days (two passages) in the absence of LIF. F: Ex-Nanog J1 cells were cultured with LY294002 for 3 or 5 days. Cells were stained for ALP activity using ALP diagnostic kit. Data represents as Mean ± SD of three independent experiments. G: Oct4 protein expression was detected by Western blot. Actin was used to verify equal loading. H: Nanog expression in Ex-Nanog J1 cells treated with LY294002 was detected by Western blot. Actin was used to verify equal loading. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Quantification of ALP colony showed that in the presence of LIF and LY294002, about 40% of wild-type J1 cells formed ALP positive colonies (LIF+ LY+). However, in the absence of LIF, the percentage of ALP positive colonies was decreased to approximate 30% (LIF− LY+). LY294002 performed similar effect on Ex-Nanog J1 cells, but the differentiated degree was slighter lower than that in wild-type J1 cells. ALP positive rates in Ex-Nanog J1 cells treated with LY294002 were, respectively, around 60% (LIF+ LY+) and 50% (LIF− LY+) in the presence or absence of LIF (Fig. 4C). Exogenous Nanog alleviated LY294002-induced differentiation, which was also confirmed by SSEA1 expression (Fig. 4D). With increased culture times, the morphology of Ex-Nanog J1 cells began to resemble Wild-type J1 cells in the presence of LY294002 alone (Fig. 4E). And, the ALP positive rate in Ex-Nanog J1 cells treated with LY294002 was significantly decreased (Fig. 4F).

We then analyzed Oct4 expression level in Ex-Nanog J1 cells. Oct4 protein was highly expressed in Ex-Nanog J1 cells in the absence of LY294002 (LIF− LY− or LIF+ LY−) and was downregulated with the treatment of LY294002 (Fig. 4G). Nanog expression level was also examined in Ex-Nanog J1 cells, which was downregulated when cells were treated with LY294002. This downregulation was partially weakened by LIF (Fig. 4H).

DISCUSSION

In this study, we present novel data about the relationship between PI3K signaling pathway and Nanog overexpression. We demonstrate that exogenous Nanog maintains mouse ES cells pluripotency in the absence of LIF, and alleviates the effect of LY294002 on ES

cells differentiation. However, exogenous Nanog is insufficient to totally reverse LY294002-induced differentiation. It has been shown that PI3K signaling pathway maintains ES cell pluripotency by directly regulating Nanog expression [Storm et al., 2007]. Our results further suggest that PI3K pathway may also contribute to inhibition of ES differentiation by other mechanism. PI3K plays a role in negative regulation of ERK activity in ES cells. The activation of ERKs appears to promote differentiation [Burdon et al., 1999]. Paling et al. [2004] shows that inhibition of PI3K signaling in mouse ES cells enhances activation of ERKs, which plays a functional role in the loss of pluripotency. The direct effect of PI3K pathway inhibition on Nanog expression can be compensated by exogenous Nanog. But the other effects of PI3K pathway inhibition cannot be replaced by Nanog overexpression. This may explain why forced Nanog overexpression cannot completely reverse mouse ES cells differentiation induced by LY294002.

Storm et al. [2007] have demonstrated that expression of an inducible form of Nanog was able to at least partially prevent LY294002-induced differentiation of mouse ES cells. In their report, 5 μM LY294002 was adopted. However, in our experiment, we observed exogenous Nanog was insufficient to reverse the differentiation when 10 μM LY294002 was used. Our results show that exogenous Nanog weaken the differentiation induced by LY294002 in ES cells. But in the presence of 10 μM LY294002, exogenous Nanog is insufficient to reverse LY294002-induced differentiation. Based Storm's and our report, we suggest PI3K signaling activation is required for maintenance of pluripotency of mouse ES cells. The effect of PI3K pathway in mouse ES cells was partially, not totally, mediated by regulation of Nanog expression.

In our experiment, we observed that LIF reduced the degree of differentiation induced by LY294002. In the presence of LIF, the protein levels of Oct4 and ALP were higher than that without LIF. Previous reports have demonstrated that gp130 may couple to the PI3K pathway, and some studies also have reported that LIF directly activates PI3K-dependent signals by increasing phosphorylation of serine 473 on Akt [Paling et al., 2004; Watanabe et al., 2006]. Thus, the effect of LY294002 can be weakened in the presence of LIF. Besides mentioned above, LIF can also directly upregulate Nanog expression. Nanog was not considered as a direct target of STAT3 when it was first identified [Chambers et al., 2003]. More recent studies, however, have presented Nanog 5′ promoter region containing STAT3 and Brachyury binding sites. In the presence of LIF, activated STAT3 interacts with Brachyury and binds to the Nanog promoter, resulting in upregulation of Nanog expression [Suzuki et al., 2006]. This may be the reason why LIF reduces the effect of LY294002 on ES cell differentiation. Therefore, in the presence of LIF and LY294002, wild-type J1 cells and Ex-Nanog J1 cells show lower differentiation degree than those in the presence of LY294002 alone.

ES cells depend on a balance between self-renewal and differentiation. Based on our findings we propose that PI3K signaling pathway plays an important role in mouse ES cell pluripotency. Inhibiting the activation of PI3K signaling pathway can skew the balance of ES cell self-renewal and differentiation, resulting in differentiation. Forced exogenous Nanog alleviates this differentiation, but is insufficient to totally reverse it. Therefore it will be interesting to further evaluate how PI3K inhibition alters the undifferentiated state of ES cells, and how Nanog maintains ES cell pluripotency.

ACKNOWLEDGMENTS

This work was supported by 863 projects from Ministry Science & Technology of China (2006AA02A110), National Natural Science Foundation of China (30570357 and 30600238) and by Tianjin Municipal Science and Technology Commission (06YFSZSF01300 and 07JCYBJC11200).

REFERENCES

Armstrong L, Hughes O, Yung S, Hyslop L, Stewart R, Wappler I, Peters H, Walter T, Stojkovic P, Evans J, Stojkovic M, Lako M. 2006. The role of PI3K/AKT, MAPK/ERK and NFkappabeta signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. Hum Mol Genet 15:1894–1913.

Burdon T, Stracey C, Chambers I, Nichols J, Smith A. 1999. Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. Dev Biol 210:30–43.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113:643–655.

Dahéron L, Opitz SL, Zaehres H, Lensch WM, Andrews PW, Itskovitz-Eldor J, Daley GQ. 2004. LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. Stem Cells 22:770–778.

Evans MJ, Kaufman MH. 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156.

Hallmann D, Trümper K, Trusheim H, Ueki K, Kahn CR, Cantley LC, Fruman DA, Hörsch D. 2003. Altered signaling and cell cycle regulation in embryonal stem cells with a disruption of the gene for phosphoinositide 3-kinase regulatory subunit p85alpha. J Biol Chem 278:5099–5108.

Hamazaki T, Kehoe SM, Nakano T, Terada N. 2006. The Grb2/Mek pathway represses Nanog in murine embryonic stem cells. Mol Cell Biol 26:7539–7549.

Humphrey RK, Beattie GM, Lopez AD, Bucay N, King CC, Firpo MT, Rose-John S, Hayek A. 2004. Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. Stem Cells 22:522–530.

Hyslop L, Stojkovic M, Armstrong L, Walter T, Stojkovic P, Przyborski S, Herbert M, Murdoch A, Strachan T, Lako M. 2005. Downregulation of NANOG induces differentiation of human embryonic stem cells to extra-embryonic lineages. Stem Cells 23:1035–1043.

Jirmanova L, Afanassieff M, Gobert-Gosse S, Markossian S, Savatier P. 2002. Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells. Oncogene 21:5515–5528.

Li ZJ, Wang ZZ, Zheng YZ, Xu B, Yang RC, Scadden DT, Han ZC. 2005. Kinetic expression of platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) during embryonic stem cell differentiation. J Cell Biochem 95:559–570.

Li J, Wang G, Wang C, Zhao Y, Zhang H, Tan Z, Song Z, Ding M, Deng H. 2007. MEK/ERK signaling contributes to the maintenance of human embryonic stem cell self-renewal. Differentiation 75:299–307.

Liu N, Lu M, Tian X, Han Z. 2007. Molecular mechanisms involved in self-renewal and pluripotency of embryonic stem cells. J Cell Physiol 211:279–286.

Liu N, Feng X, Fang Z, Ma F, Lu S, Lu M, Han Z. 2008. Identification of genes regulated by nanog which is involved in ES cells pluripotency and early differentiation. J Cell Biochem 104:2348–2362.

Matsuda T, Nakamura T, Nakao K, Arai T, Katsuki M, Heike T, Yokota T. 1999. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. EMBO J 18:4261–4269.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113:631–642.

Miyabayashi T, Teo JL, Yamamoto M, McMillan M, Nguyen C, Kahn M. 2007. Wnt/beta-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency. Proc Natl Acad Sci USA 104:5668–5673.

Niwa H, Burdon T, Chambers I, Smith A. 1998. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev 12:2048–2060.

Paling NR, Wheadon H, Bone HK, Welham MJ. 2004. Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. J Biol Chem 279:48063–48070.

Pyle AD, Lock LF, Donovan PJ. 2006. Neurotrophins mediate human embryonic stem cell survival. Nat Biotechnol 24:344–350.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. 2000. Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. Nat Biotechnol 18:399–404.

Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3- specific inhibitor. Nat Med 10:55–63.

Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336:688–690.

Storm MP, Bone HK, Beck CG, Bourillot PY, Schreiber V, Damiano T, Nelson A, Savatier P, Welham MJ. 2007. Regulation of Nanog expression by phosphoinositide 3-kinase-dependent signaling in murine embryonic stem cells. J Biol Chem 282:6265–6273.

Suzuki A, Raya A, Kawakami Y, Morita M, Matsui T, Nakashima K, Gage FH, Rodríguez-Esteban C, Izpisúa Belmonte JC. 2006. Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. Proc Natl Acad Sci USA 103:10294–10299.

Takahashi K, Murakami M, Yamanaka S. 2005. Role of the phosphoinositide 3-kinase pathway in mouse embryonic stem (ES) cells. Biochem Soc Trans 33:1522–1525.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147.

Vlahos CJ, Matter WF, Hui KY, Brown RF. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4- one (LY294002). J Biol Chem 269:5241–5248.

Watanabe S, Umehara H, Murayama K, Okabe M, Kimura T, Nakano T. 2006. Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. Oncogene 25:2697–2707.

Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA. 2005. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2:185–190.

Ying QL, Nichols J, Chambers I, Smith A. 2003. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115:281–292.