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Injectoassay for Functional Activity of *Nanog* to Maintain the Undifferentiated State of Embryonic Stem Cells

Mikako Saito*, Tsukasa Kaeriyama, Masato Koyama and Hideaki Matsuoka

Tokyo University of Agriculture and Technology, Department of Biotechnology and Life Science, 2-24-16, Naka-cho, Koganei, Tokyo 184-8588, Japan

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Nanog is one of key genes in the complicated gene network maintaining the undifferentiated state of embryonic stem (ES) cells. Injectoassay was applied to the evaluation of the functional activity of *Nanog* in viable ES single-cells. The functional sequence of *Nanog* was cloned from the genome DNA of a mouse ES cell and inserted into an overexpression vector together with a reporter gene of green fluorescent protein. The thus-prepared *Nanog* expression vector was injected into mouse ES single-cells in a semi-quantitative manner using a sub-micropipette containing a defined vector concentration. Culture was continued for 72 h in the presence or absence of the leukemia inhibitory factor (LIF) and the morphological changes of colonies were analyzed to distinguish the differentiated state from the undifferentiated state. When the *Nanog* expression level was sufficiently high, the undifferentiated state could be maintained for 48 h, even in the absence of LIF.

1. Introduction

In regenerative engineering, it is currently required to develop an experimental method that can analyze the cell–cell communication between undifferentiated and differentiated cells in the viable state. A promising strategy is to apply a differentiation-associated factor only to a target single-cell and to measure the dynamic response of a neighboring single-cell as well as of the target single-cell itself. Their response includes morphological changes and cell growth, the expression of relevant genes, and intercellular molecular movement. To meet this requirement, we developed injectoassay based on femtoinjection technology.[1]

Practically, embryonic stem (ES) cells can be maintained in the undifferentiated state in a culture medium containing the leukemia inhibitory factor (LIF). In response to the

*Corresponding author: e-mail: mikako@cc.tuat.ac.jp

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extracellular signal of LIF, intracellular signal transduction occurs and activates various factors that are linked to each other via feedback and feedforward networks.[2] Typical factors are transcription factors, such as *Stat3*,[3] *Oct3/4*,[4,5] *Sox2*,[6] *Nanog*,[7] *Cdx2*,[8] and *Gata6*.[9] Before analyzing such a complicated network in viable single-cells, it is necessary to investigate the gene expression controllability of each factor and its effects on the maintenance of the undifferentiated state or on the initiation of differentiation.

Here, we have selected *Nanog* because of its strong potential to maintain the undifferentiated state of ES cells. We expected to fix ES cells in the undifferentiated state by increasing the expression level of *Nanog*.

2. Materials and Methods

2.1 *ES cell culture*

EB3, a clone of feeder-free mouse ES cells, was provided by H. Niwa (Center for Developmental Biology, RIKEN, Kobe, Japan) and cultured at 37 °C in the absence of feeder cells in GMEM (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10^{-4} M 2-mercaptoethanol, 1× nonessential amino acids, and 1000 U/ml LIF on gelatin-coated dishes.[10]

2.2 *Construction of a* Nanog *expression vector*

RNA was extracted from EB3 cells and applied to reverse transcription to prepare cDNA. Then, cDNA corresponding to *Nanog* was amplified by polymerase chain reaction (PCR) using the following primer set designed according to the database of mouse *Nanog*:

Nan(F): 5'- GAAATCCCTTCCCTCGCCAT -3',

Nan(R): 5'- ACTTACGCAACATCTGGGCT -3'.

The PCR product was purified using GENECLEAN II KIT (BIO101 Systems). The purified cDNA was inserted into a plasmid pCR2.1 by TA cloning and then introduced into competent cells, *Escherichia coli* DH5α, by the calcium method. After culture on LB agar plates containing ampicillin at 37 °C overnight, β-Gal negative colonies were picked up and the plasmid was isolated. The sequence of the insert was amplified by PCR using the following universal primer set:

Uni(F): 5'- CAGGAAACAGCTATGAC -3',

Uni(R): 5'- GTAAAACGACGGCCAG -3'.

The PCR product was analyzed with a 3100-Avant Genetic Analyzer (Applied Biosystems) and referred to the database in BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The PCR product showed 100% homology (917/917). Thus, a *Nanog* overexpression vector (pCAG-*Nanog*-IRES-EGFP) was successfully constructed (Fig. 1).

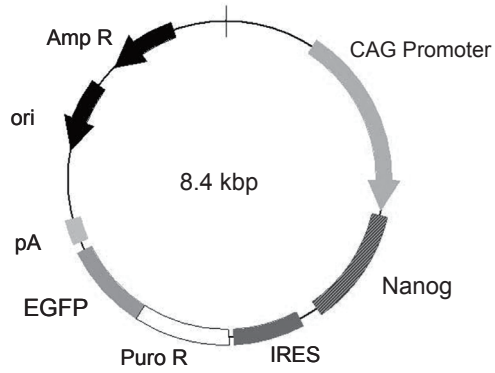


Fig. 1. Construct of a *Nanog* overexpression vector labelled with green fluorescent protein reporter gene.

2.3 *Lipofection*

GMEM containing 30 v/v% lipofectamine 2000 (Invitrogen) was mixed with the same volume of pCAG-*Nanog*-IRES-EGFP solution (5 μg DNA + 45 μl GMEM) and then added to a 0.5 ml suspension of EB cells. After mixing, the cell suspension was allowed to stand for 25 min at 25 °C. Then the cells were rinsed with PBS and suspended in 0.5 ml of GMEM for culture at 37 °C.

2.4 *Semiquantitative femtoinjection*

Glass capillaries (BF100-78-10, Sutter Instrument Co.) were pulled with a laser puller (P-2000, Sutter Instrument Co.). On the basis of previous test results,[11] the heating and pulling conditions were adjusted so that the tip diameter would be in the range of 0.6–0.8 μm. The performance of semiquantitative introduction by femtoinjection was confirmed by preliminary test injections using capillaries containing various vector concentrations in the range of 100–1000 ng/μl. The test injection was repeated until at least 2 successful gene expressions were observed for the respective concentrations.

2.5 *Confocal fluorescent microscopy*

After the femtoinjection of pCAG-*Nanog*-IRES-EGFP, ES cells were cultured at 37 °C for 24 h and then observed with a confocal laser scanning microscope (LSM510, Carl Zeiss Co., Ltd.) to acquire EGFP fluorescence images (Ex/Em: 488/507 nm). When fluorescent cells were observed, the medium was replaced by a fresh medium containing no LIF (LIF− medium). During successive culture, the fluorescence images were recorded at 48 and 72 h.

2.6 *Indicator of morphological change of colonies*

An ES single-cell is spherical and forms a spherical colony in the undifferentiated state. However, it will become flat with a complicated outline as its differentiation

proceeds. To evaluate the effect of regulation factors on the differentiation, we introduced a quantitative indicator on the basis of this morphological change. When the 2-dimensional image of a sphere is a circle, the perimeter-to-radius ratio (P/R ratio) is $2\pi r/r = 2\pi = 6.28$. When this shape becomes more complex, the P/R ratio becomes greater than 6.28, where an equivalent radius is given by the formula $\sqrt{S/\pi}$, where S is area encircled by the perimeter. The P/R value was then normalized by that of a circle, i.e., $P/R/6.28$. According to the previous report about *Oct3/4*,[12] the $P/R/6.28$ criterion for the undifferentiated state was no higher than 1.3 during the 72 h culture period.

3. Results

3.1 *Morphological changes observed during culture under various conditions*

pCAG-*Nanog*-IRES-EGFP was introduced into ES cells by lipofection and its effects on the maintenance of the undifferentiated state were investigated. After culturing at 37 °C for 24 h, the medium containing LIF was changed for a fresh medium containing no LIF and then cultured for 48 h successively. The colony became flat with a complicated outline (Fig. 2, LIF−). In contrast, the cells cultured in a medium containing LIF for 72 h maintained

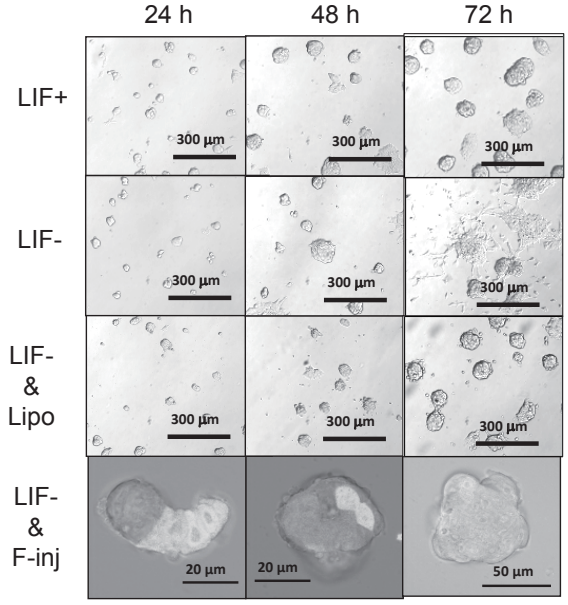


Fig. 2. Images of single-cells and colonies. LIF+: cultured in LIF+ medium throughout 0–96 h period, LIF−: cultured in LIF+ medium for initial 24 h and then in LIF− medium for 48 h, LIF−&Lipo: cultured in LIF+ medium containing *Nanog* expression vector and lipofectamine for initial 24 h and then in LIF− medium containing *Nanog* vector and lipofectamine for 48 h, LIF−&F-inj: femtoinjection of *Nanog* vector at 0 h and cultured in LIF+ medium for initial 24 h and then cultured in LIF− medium for 48 h.

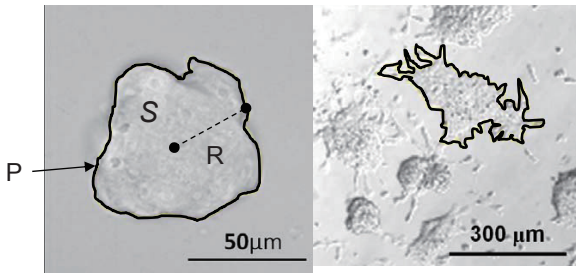
their spherical shape (Fig. 2, LIF+). On the other hand, the cells cultured in the LIF− medium in the presence of pCAG-*Nanog*-IRES-EGFP and lipofectamine maintained their spherical shape until the end of the 72 h culture period (Fig. 2, LIF−&Lipo). Therefore, *Nanog* introduced by lipofection was effective for the maintenance of the undifferentiated state even in the absence of LIF.

Next, pCAG-*Nanog*-IRES-EGFP was introduced by femtoinjection using a sub-micropipette containing a vector concentration higher than 500 ng/μl. The femtoinjection into ES single-cells was conducted at 0 h and then culture was continued for 24 h in the presence of LIF. At 24 h, some colonies emitted the fluorescence of EGFP, a reporter of *Nanog* gene expression. Those fluorescent colonies were successively cultured for 48 h in the absence of LIF. Consequently, those colonies maintained their spherical shape until the end of culture (Fig. 2, LIF−&F-inj).

3.2 *Quantitative effects of pCAG-*Nanog*-IRES-EGFP on the maintenance of undifferentiated state*

The colonies selected from the imaging data at 72 h in Fig. 2 were analyzed using an image analyzing program, Image-J. A colony obtained under LIF−&F-inj culture conditions maintained its spherical shape, and *P*/*R*/6.28 was 1.12 [Fig. 3(a)], while a colony obtained under LIF− culture conditions became flat with a complicated outline, and *P*/*R*/6.28 was 3.48 [Fig. 3(b)].

The same analysis was applied to the images of other arbitrarily selected colonies. In the presence of LIF, *P*/*R*/6.28 remained at 1.23–1.25 (Fig. 4). In contrast, in the absence



(a) (b)

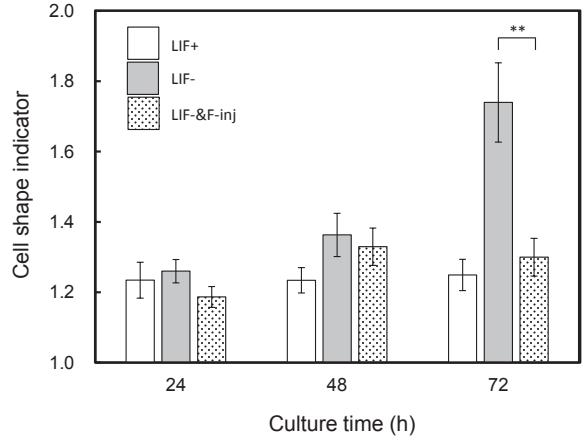


Fig. 3 (left). Examples of *P*/*R*/6.28 values. (a) Undifferentiated colony in a panel of LIF− at 72 h in Fig. 2, *P* = 308.9 μm, *R* = 43.9 μm, and *P*/*R*/6.28 = 1.12. (b) Undifferentiated colony in a panel of LIF−&F-inj in Fig. 2, *P* = 1054.9 μm, *R* = 48.3 μm, and *P*/*R*/6.28 = 3.48.

Fig. 4 (right). Quantitative effect of pCAG-*Nanog*-IRES-EGFP on the morphological changes of colonies. Refer to the legend for Fig. 3 about LIF+, LIF−, and LIF−&F-inj. Error bars: mean ± SEM for *n* = 12 (LIF+), 27 (LIF−), and 8 (LIF−&F-inj). **: $p < 0.005$.

of LIF, it increased markedly and reached 1.74 at 72 h. On the other hand, the *Nanog* vector was effective for maintaining this value at 1.30 at 72 h. According to the criterion described in § 2.6, this value indicates the undifferentiated state. The one tailed t-test showed that the difference between LIF− at 72 h and LIF−&F-inj at 72 h was statistically significant at $p < 0.005$.

4. Discussion

The gene network regulating the maintenance of the undifferentiated state and the initiation of differentiation comprises a number of factors. The involvement of *Nanog* in this regulation network is roughly depicted in Fig. 5. According to this scheme, several factors including *Nanog* may maintain the undifferentiated state even in the absence of LIF, which is practically used for the maintenance of the undifferentiated state from outside of the cell. However, the regulation conditions of respective factors are not clear.

In the case of *Oct3/4*, for instance, its expression is observed in the undifferentiated state of ES cells. When the level of its expression increases, the cells differentiate into endodermal cells. On the other hand, when the level decreases, the cells differentiate into trophectodermal cells. Experimentally, we could initiate the differentiation by the femtoinjection of a high concentration of an *Oct3/4* overexpression vector into mouse ES cells in the presence of LIF.[12] According to the network scheme, however, the increase in the level of *Oct3/4* expression might induce the up-regulation of *Nanog* and consequently set the cells in the undifferentiated state. Therefore, our result seemed to be paradoxical. These implies the importance of quantitative discussion about the relative expression levels of multiple factors.

These results suggest that the expression of *Nanog* at a sufficiently high level is predominant over the LIF and the intrinsic expression level of *Oct3/4* for the maintenance of the undifferentiated state. The next step should be the quantitative analyses of relative

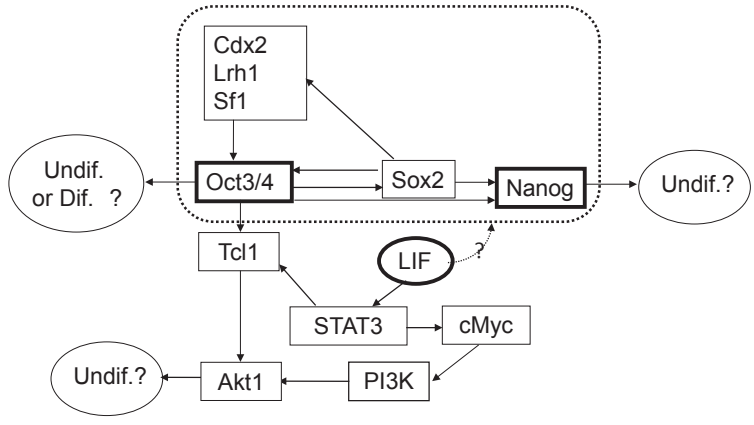


Fig. 5. Possible involvement of *Nanog* in the regulation of the undifferentiated state of ES cells.

expression levels of multiple factors at a single-cell level. Injectoassay is expected to be a useful way to conduct such analyses. Those analyses may lead us to the final goal of the elucidation of the regulation network mechanism in ES cells.

5. Conclusion

The overexpression of *Nanog* in viable mouse ES single-cells could maintain the undifferentiated state of the cells even in the absence of LIF. This result indicates that the same method can be applied to other relevant factors. The next step is to apply this method to the analysis of the simultaneous behavior of multiple factors relevant to *Nanog* and *Oct3/4*.

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References

1 H. Matsuoka, M. Saito and H. Funabashi: Embryonic Stem Cells—Basic Biology to Bioengineering, ed. M. S. Kallos (InTech, Rijeka, Croatia, 2011) p. 149.
2 V. Chickarmane, C. Troein, U. A. Nuber, H. M. Sauro and C. Peterson: PLoS Computational Biology **2** (2006) e123.
3 T. Matsuda, T. Nakamura, K. Nakao, T. Arai, M. Katsuki, T. Heike and T. Yokota: EMBO J. **18** (1999) 4261.
4 H. Niwa, J. Miyazaki and A. G. Smith: Nat. Genet. **24** (2000) 372.
5 M. Nishimoto, S. Miyagi, T. Yamagishi, T. Sakaguchi, H. Niwa, M. Muramatsu and A. Okuda: Mol. Cell Biol. **25** (2005) 5084.
6 A. A. Avilion, S. K. Nicolis, L. H. Pevny, L. Perez, N. Vivian and R. Lovell-Badge: Genes Dev. **7** (2003) 126.
7 S. Mora-Castilla, J. R. Tejedo, R. Tapia-Limonchi, I. Díaz, A. B. Hitos, G. M. Cahuana, A. Hmadcha, F. Martín, B. Soria and F. J. Bedoya: Stem Cells Int. **2014** (2014) doi. org/10.1155/2014/379678.
8 A. K. S. Roman, B. E. Aronson, S. D. Krasinski, R. A. Shivdasani and M. P. Verzi: J. Biol. Chem. **290** (2015) 1850.
9 J. Fujikura, E. Yamato, S. Yonemura, K. Hosoda, S. Masui, K. Nakao, J. Miyazaki and H. Niwa: Genes Dev. **16** (2002) 784.
10 A. G. Smith: J. Tissue Culture Methods **13** (1991) 89.
11 H. Matsuoka, S. Shimoda, M. Ozaki, H. Mizukami, M. Shibusawa, Y. Yamada and M. Saito: Biotechnol. Lett. **29** (2007) 341.
12 H. Matsuoka and M. Saito: ECS Trans. **16** (2009) 9.