# Effects of Extracellular Signaling on the Endogenous Expression of Self-Renewal-Stimulating Factor Genes in Mouse Embryonic Stem Cells

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## ABSTRACT

**In order to provide the basis for developing practical mouse embryonic stem cells (mESCs) culture method, how the endogenous level of self-renewal-stimulating factor genes was altered in the mESCs by different extracellular signaling was investigated in this study. For different extracellular signaling, mESCs were cultured in 2 dimension (D), 3D and integrin-stimulating 3D culture system in the presence or absence of leukemia inhibitory factor (LIF) and transcriptional level of *Lif*, *Bmp4* and *Wnt3a* was evaluated in the mESCs cultured in each system. The expression of three genes was significantly increased in 3D system relative to 2D system under LIF-containing condition, while only *Wnt3a* expression was increased by 3D culture under LIF-free condition. Stimulation of integrin signaling in mESCs within 3D system with exogenous LIF significantly up-regulated transcriptional level of *Bmp4*, but did not induce transcriptional regulation of *Lif* and *Wnt3a*. In the absence of LIF inside 3D system, the expression of *Lif* and *Bmp4* was significantly increased by integrin signaling, while it significantly decreased *Wnt3a* expression. Finally, the signal from exogenous LIF significantly caused increased expression of *Lif* in 2D system, decreased expression of *Bmp4* in both 2D and 3D system, and decreased expression of *Wnt3a* in integrin-stimulating 3D system. From these results, we identified that endogenous expression level of self-renewal-stimulating factor genes in mESCs could be effectively regulated through artificial and proper manipulation of extracellular signaling. Moreover, synthetic 3D niche stimulating endogenous secretion of self-renewal-stimulating factors will be able to help develop growth factor-free maintenance system of mESCs.**

**(Key words : Mouse Embryonic stem cells, Self-renewal-stimulating factor, Endogenous gene expression, Extracellular signaling)**

## INTRODUCTION

Embryonic stem cells (ESCs) derived from inner cell mass of blastocyst are pluripotent cells which can proliferate indefinitely without senescence in the undifferentiated state and differentiate into three-germ layer-specific tissue cells. For therapeutic purpose of damaged cells, tissues and organs, pluripotency of ESCs should be necessarily maintained during mass production of highly qualified ESCs. To date, Stat3 (Boeuf *et al.*, 1997; Niwa *et al.*, 1998; Matsuda *et al.*, 1999), β-catenin (Sato *et al.*, 2004; Miyabayashi *et al.*, 2007) and Smad 1/5/8 (Ying *et al.*, 2003) have been reported as key signal molecules supporting mouse ESC self-renewal, and leukemia inhibitory factor (LIF), bone morphogenetic protein 4 (Bmp4) and wingless-type MMTV integration site family, member 3a (Wnt3a) known as stimulator of Stat3, β-catenin and Smad 1/5/8 signal proteins, respectively, have been essentially employed to *in vitro* maintenance of ESC pluripotency. However, the high cost as well as irregular exogenous effects of these self-renewal-stimulating factors may prevent lots of researchers from harvesting a sufficient quantity of undifferentiated ESCs and the widespread adjustment of ESCs to basic research and clinical application may be greatly limited by these issues.

Recently, new cell-friendly tool making it possible three dimension (D) culture of cells and providing specific extracellular signaling to cells inside 3D matrix was developed and successfully applied to 3D feeder-free culture of mouse ESCs (mESCs) by constructing biomimetic microenvironment alternating extracellular matrix of mouse embryonic fibroblasts (Lee *et al.*, 2010). The

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results from this new system for culturing mESCs showed that cell fate decision could be sufficiently controlled by diverse extracellular signaling and also suggested a possibility that secretion of ESC self-renewal-stimulating paracrine factors may be sufficiently regulated by artificial external stimulation.

Therefore, in this study, in order to investigate how diverse extracellular stimulation regulates the expression of major self-renewal-stimulating factor genes in mESCs, the endogenous expression levels of *Lif*, *Bmp4* and *Wnt3a* were evaluated by quantitative real-time PCR in the mESCs cultured under diverse extracellular signaling. The 2D, 3D and integrin-stimulating 3D culture system in the presence or absence of intracytoplasmic Stat3 signaling induced by exogenous LIF were used to induce different extracellular signaling in mESCs, and resultant expression values of three genes were compared among them.

## MATERIALS AND METHODS

### Culture of Mouse Embryonic Stem Cells (mESCs)

The mESC E14tg2a line (ATCC, Manassas, VA) were cultured on 10 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, MO)-inactivated mouse embryonic fibroblasts (MEFs) in standard mESC culture medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, Grand Island, NY) supplemented with 15% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 0.1 mM β-mercaptoethanol (Gibco Invitrogen), 1% (v/v) nonessential amino acids (NEAA; Gibco Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich), 2 mM L-glutamine (Gibco Invitrogen), a 1% (v/v) lyophilized mixture of penicillin and streptomycin (Gibco Invitrogen) and 1,000 units/ml mouse LIF (Chemicon International, Temecula, CA). Subpassage of mESCs was conducted every 3 day and fresh medium was exchanged daily during subculture.

### Adhesion Peptide Synthesis and Purification

According to protocol described previously (Lutolf *et al.*, 2001; Lutolf and Hubbell, 2003), an automated peptide synthesizer (Chemspeed, Augst, Switzerland) adjusting Fmoc/HBTU/HOBT chemistry was used for synthesis of peptides on solid resin (Novasyn TGR, Novabiochem, Laeufelfingen, Switzerland) and C18 chromatography (Biocad 700E; PerSpective Biosystems, Farmington, MA) was employed for purification of synthesized peptide. Subsequently, synthetic peptide sequence was identified by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry. Sequences of all synthesized peptides were AcGCGWGRGDSPG, GCRDTTSWSQG and AcGCRRRRDRDAEIDGIELG.

### Formation of Poly(Ethylene Glycol) (PEG)-Based Hydrogel and Conjugation of Adhesion Peptides

Firstly, 3 arm-PEG was functionalized with vinylsulfone (Sigma-Aldrich) as described previously (Lutolf *et al.*, 2001). Then, for making PEG-based hydrogel, vinylsulfone-functionalized 3 arm-PEG (PEG-VS) was conjugated with dicystein-including peptides with an intervening matrix metalloproteinase (MMP)-specific cleavage site (herein referred to as crosslinker) through a Michael-type addition reaction (Lutolf and Hubbell, 2003). Moreover, internal mechanical property derived from 10% (w/v) PEG-hydrogel with 0.7 stoichiometric ratio (the ratio of vinylsulfone to thiol group) was adjusted to entire experiments. Incorporation of specific integrin-stimulating adhesion peptides into the PEG-hydrogel was conducted by mixing the precursor solution of PEG-VS dissolved in mESC culture medium with the precursor solution of adhesion peptides dissolved in pH 8.0 0.3M HEPES buffer and incubating for 30 minutes at room temperature.

### Incorporation of mESCs into PEG-hydrogel and Culture

As previously described protocol (Lee *et al.*, 2010), precursor solution of PEG-VS conjugated without or with adhesion peptides was simultaneously mixed with crosslinker dissolved in pH 8.0 0.3M HEPES buffer and mESC suspended in mESC culture medium. Subsequently, circular disks were derived from 6 μl of the mixed precursor solution, gelation of these occurred within 30 minutes at 37℃, and the mESCs inserted into PEG-hydrogel were cultured for 5 days in LIF-containing or -free standard mESC culture medium. After culture, mESCs were retrieved from PEG-hydrogel by incubating in 10 mg/ml collagenase I (Sigma-Aldrich) solution for 10 minutes.

### Analysis of Relative Gene Expression

Relative expression of genes was analyzed by quantitative real-time PCR. According to manufacturer's instruction, the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) was used for extracting total mRNA from samples and a Reverse Transcription System (Promega, Madison, WI) for synthesizing cDNA from extracted mRNA. Subsequently, the quantitative real-time PCR was conducted with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA) under the Bio-Rad iCycler iQ system (Bio-Rad Laboratories). Melting curve data was measured for identifying PCR specificity and β-actin mRNA level for normalizing the specific gene expression. Relative mRNA level was presented as $2^{-\Delta\Delta Ct}$, where Ct=threshold cycle for target amplification, $\Delta Ct = Ct_{\text{target gene}}$ (specific genes for each sample)$-Ct_{\text{internal reference}}$ (*β-actin* for each sample), and $\Delta\Delta Ct = \Delta Ct_{\text{sample}}$ (treatment sample in each experiment)$-\Delta Ct_{\text{calibrator}}$ (con-

**Table 1. Oligonucleotide primers and PCR cycling conditions.**

| Genes | GenBank number | Primer sequence | | Size (bp) | Temperature (℃) |
|---|---|---|---|---|---|
| | | Sense (5′>3′) | Anti-sense (5′>3′) | | |
| β-actin | X03672 | TACCACAGGCATTGTGATGG | TCTTTGATGTCACGCACGATT | 200 | 60 |
| *Lif* | M63419.1 | GACCAATATCACCCGGGACCA | CACGTGGCCCACACGGTACT | 153 | 60 |
| *Bmp4* | BC013459 | TTGTGTCCCCACTGAACTGA | TTGAATGGGAACGTGTGTGT | 198 | 60 |
| *Wnt3a* | NM_009522.2 | AAGACCTGCTGGTGGTCGCA | GACCAGGTCGCGTTCTGTCG | 183 | 60 |

trol sample in each experiment). Table 1 described general information and sequences of primers designed with cDNA sequences derived from GenBank for mouse and by Primer3 software (Whitehead Institute/MIT Center for Genome Research).

### Statistical Analysis

The statistical analysis of all the numerical data shown in each experiment was performed using the Statistical Analysis System (SAS) program. Moreover, in detecting a significance of the main effects through variance (ANOVA) analysis in the SAS package, each treatment was compared by the least-square or DUNCAN methods and the less than 0.05 of $p$ value was regarded as significant differences.

## RESULTS

### Effects of 3D or 2D Culture System on the Endogenous Expression of Self-Renewal-Stimulating Factor Genes in mESCs

To evaluate the effects of 3D culture system on the endogenous transcriptional regulation of self-renewal-

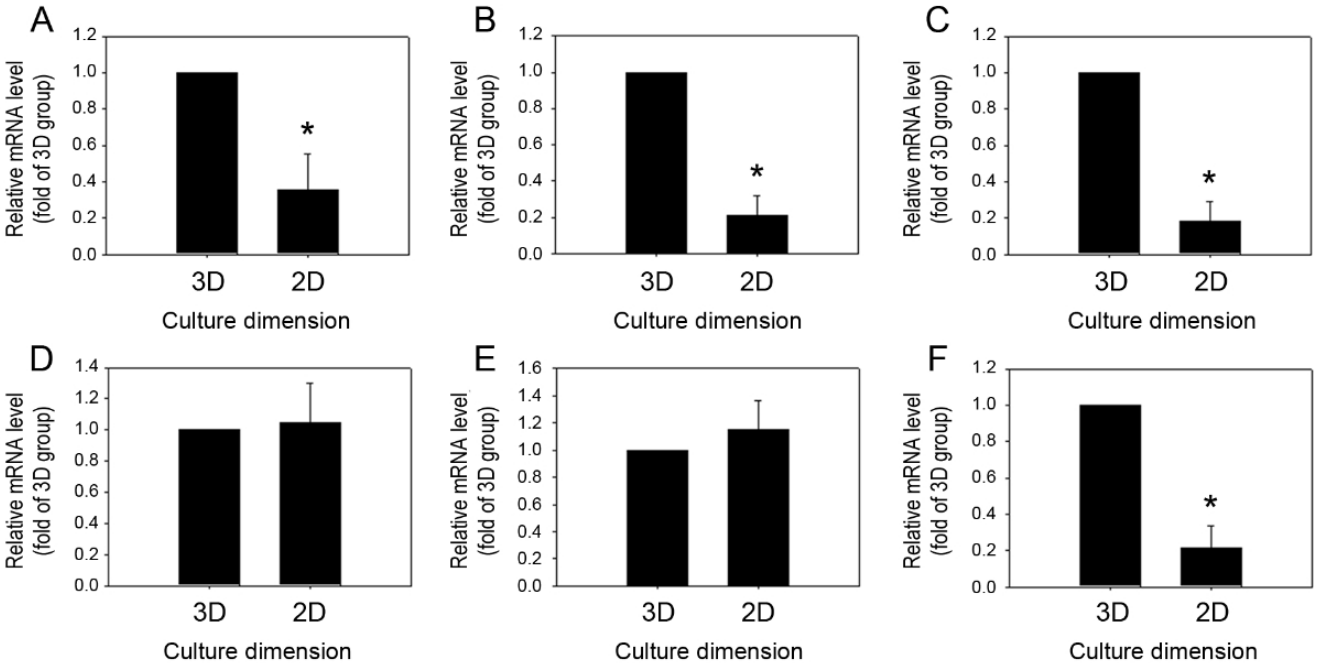


**Fig. 1. Effects of 3 dimension (D) or 2D culture system on the expression of self-renewal-stimulating factor genes in the mouse embryonic stem cells (mESC) in the presence or absence of leukemia inhibitory factor (LIF).** For the construction of 3D system, vinyl sulfon-functionalized 3-arm PEG was conjugated with matrix metalloproteinase (MMP)-sensitive crosslinker in 0.7 stoichiometric ratio, and the 2D system was constructed by tissue culture dish coated with nothing. After 5 days culture of mESC in the presence or absence of LIF, analysis of *Lif*, *Bmp4* and *Wnt3a* expression were conducted by real-time PCR. As the results, in the presence of LIF (A-C), 3D system showed significant up-regulation of *Lif* (A), *Bmp4* (B) and *Wnt3a* (C). In case of LIF-free condition (D-F), significant up-regulation of *Wnt3a* (F) was observed in mESC cultured in 3D system, compared to those in 2D system. Whereas, no significant difference in *Lif* (D) and *Bmp4* (E) expression was detected between 3D and 2D system. All data derived from real-time PCR are means±s.d. of five independent experiments. *$p$<0.05.

stimulating factor genes in mESCs, transcriptional level of *Lif*, *Bmp4* and *Wnt3a* was compared between mESCs cultured on 2D and inside 3D system in the presence or absence of LIF. As the results, in the presence of Stat3 activation by LIF, 3D system showed significant transcriptional up-regulation of all self-renewal-stimulating factors examined (Fig. 1A~C). Whereas, in the absence of LIF, no significant differences in the transcriptional regulation of *Lif* (Fig. 1D) and *Bmp4* (Fig. 1E) was detected between mESCs cultured inside 3D and on 2D system, and 3D system induced significant transcriptional up-regulation of *Wnt3a* (Fig. 1F).

**Effects of Integrin Signaling inside 3D Culture System on the Endogenous Expression of Self-Renewal-Stimulating Factor Genes in mESCs**

For activating integrin $\alpha_5\beta_1$, $\alpha_v\beta_5$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$ expressed on mESCs (Lee *et al.*, 2010), three integrin-stimulating adhesion peptides were incorporated to 3D system and transcriptional level of *Lif*, *Bmp4* and *Wnt3a* in the mESCs cultured inside integrin-stimulating 3D system were evaluated and compared with tho- se inside 3D system without any integrin stimulation in the presence or absence of Stat3 activation by LIF. Under LIF-containing condition, integrin activation of mESCs within 3D system induced significant transcriptional up-regulation of *Bmp4*, while significant alteration of *Lif* and *Wnt3a* transcriptional levels were not induced, in spite of integrin signaling within 3D system (Fig. 2A~C). Under LIF-free condition inside 3D system, integrin signaling significantly up-regulated transcriptional level of *Lif* (Fig. 2D) and *Bmp4* (Fig. 2E) in mESCs and significantly down-regulated transcriptional level of *Wnt3a* (Fig. 2F).

**Effects of Stat3 Signaling under 2D, 3D and Integrin-Stimulating 3D Culture System on the Endogenous Expression of Self-Renewal-Stimulating Factor Genes in mESCs**

Next, we investigated how Stat3 signaling induced by exogenous LIF influences transcriptional regulation of self-renewal-stimulating factor genes in mESCs under three different culture systems. In 2D system, exogenous LIF significantly down-regulated transcriptional level of *Lif* (Fig. 3A), significantly up-regulated that of *Bmp4* (Fig. 3B), and showed no significant difference in that of *Wnt3a* (Fig. 3C). In case of 3D system, only

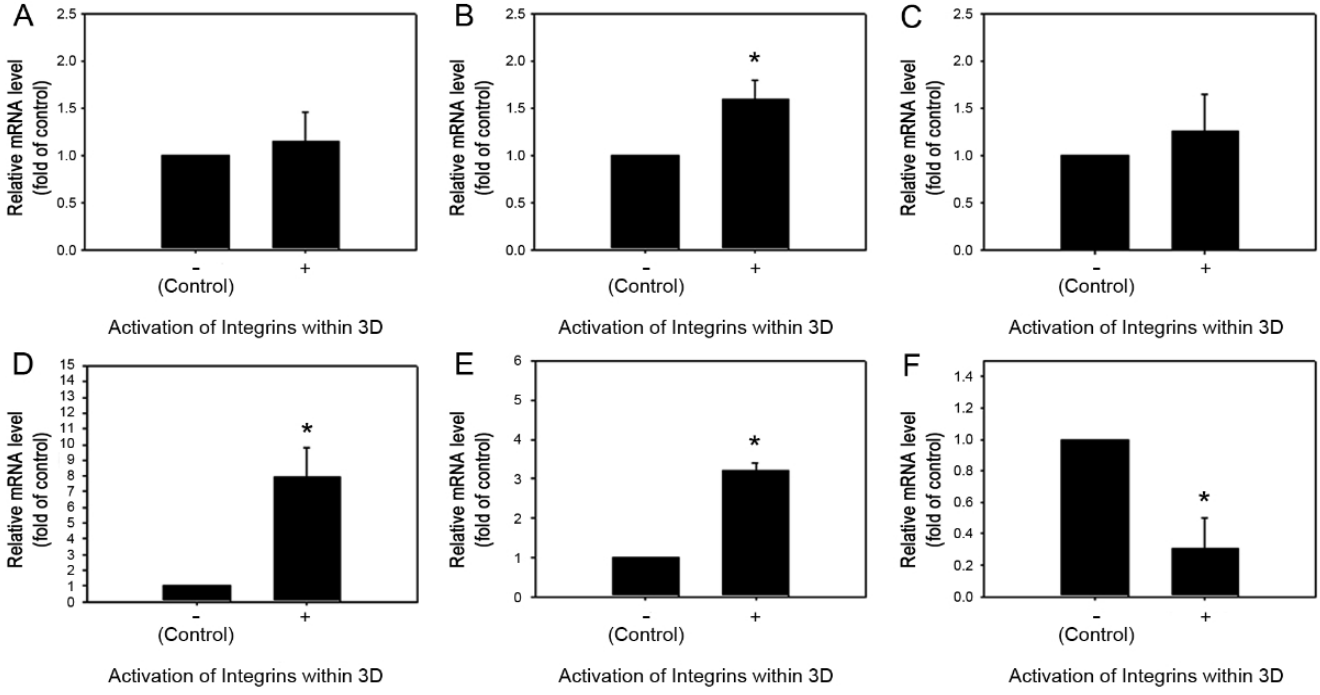


**Fig. 2. Effects of integrin activation inside 3 dimension (D) culture system on the expression of self-renewal-stimulating factor genes in the mouse embryonic stem cells (mESC) in the presence or absence of leukemia inhibitory factor (LIF).** The 3D system was constructed by the conjugation of vinyl sulfon-functionalized 3-arm PEG with matrix metalloproteinase (MMP)-sensitive crosslinker in 0.7 stoichiometric ratio, and integrin heterodimers were stimulated by encapsulating mESCs into 3D PEG-based scaffold ($r$=0.7) conjugated with 400 μM RGDSP (for co-activating integrin $\alpha_5\beta_1$ and $\alpha_v\beta_5$), 800 μM TTSWSQ (for activating integrin $\alpha_6\beta_1$) and 800 μM AEIDGIEL (for activating integrin $\alpha_9\beta_1$) adhesion peptides. The expression of *Lif*, *Bmp4* and *Wnt3a* from mESC cultured for 5 days in the presence or absence of LIF was analyzed by real-time PCR. In the presence of LIF (A-C), integrin activation inside 3D matrix caused no significant difference in *Lif* (A) and *Wnt3a* (C) expression, but it significantly up-regulated *Bmp4* expression (B). In the absence of LIF (D-F), the expression of *Lif* (D) and *Bmp4* (E) genes was significantly increased by integrin stimulation inside 3D matrix, while the expression of *Wnt3a* was significantly decreased (F). All data derived from real-time PCR are means±S.D. of five idependent experiments. $^*p$<0.05.

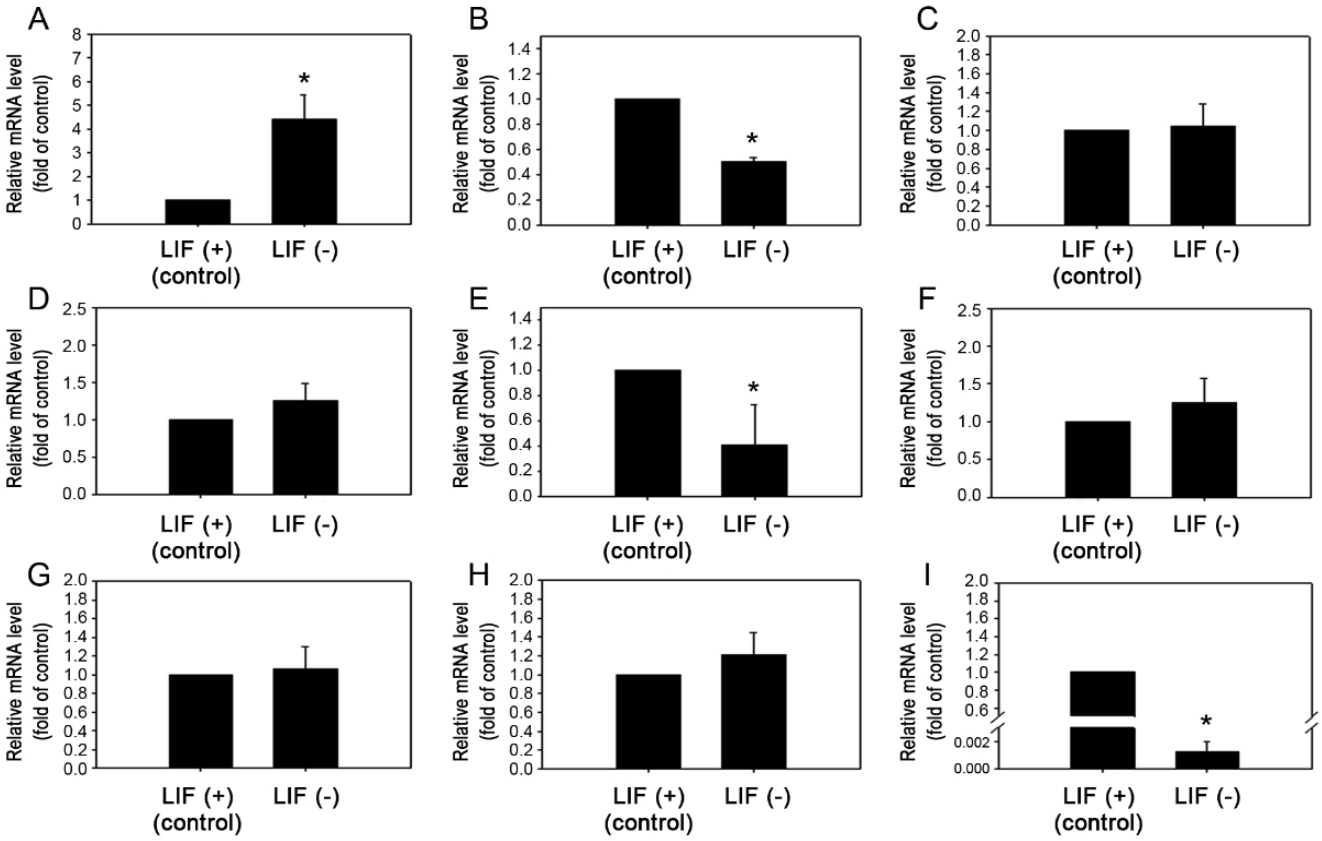


**Fig. 3. Effects of exogenous leukemia inhibitory factor (LIF) under 2 dimension (D), 3D and integrin-stimulating 3D culture system on the endogenous level of self-renewal-stimulating factor genes in the mouse embryonic stem cells (mESC).** The tissue culture dish coated without anything were prepared for the construction of 2D culture system and the conjugation of vinyl sulfon-functionalized 3-arm PEG with matrix metalloproteinase (MMP)-sensitive crosslinker in 0.7 stoichiometric ratio were conducted for the construction of 3D culture system. Moreover, the 3D PEG-based scaffolds ($r$=0.7) incorporating 400 μM RGDSP, 800 μM TTSWSQ, and/or 800 μM AEIDGIEL were employed for co-activation of integrin $\alpha_5\beta_1$, $\alpha_v\beta_5$, $\alpha_6\beta_1$, $\alpha_9\beta_1$ in the mESC. Transcriptional level of the mESC on 2D culture system or inside 3D and integrin-stimulating 3D culture system were estimated by real-time PCR at 5 days culture in the presence or absence of LIF. Under 2D culture system (A~C), LIF supplementation induced significant inhibition of *Lif* expression (A), significant stimulation of *Bmp4* expression (B), and no significant difference in *Wnt3a* expression (C) in cultured mESCs. In case of 3D culture system (D~F), significant stimulation of transcriptional level by exogenous LIF was only observed in *Bmp4* (E) and, in the mESCs cultured inside integrin-stimulating 3D culture system (G~I), significant increase of *Wnt3a* expression (I) was caused by exogenous LIF . All data derived from real-time PCR are means±S.D. of five independent experiments. $^{*}p<0.05$.

*Bmp4* showed significant transcriptional up-regulation resulted from exogenous LIF (Fig. 3E) and transcriptional level of the other genes, *Lif* (Fig. 3D) and *Wnt3a* (Fig. 3F), did not show significant differences by exogenous LIF. Finally, in mESCs experiencing integrin stimulation inside 3D system, exogenous LIF provoked no significant difference in the regulation of endogenous *Lif* (Fig. 3G) and *Bmp4* (Fig. 3H) transcriptional level, while it induced significant up-regulation of endogenous *Wnt3a* transcriptional level (Fig. 3I).

## DISCUSSION

In this study, we could identify that endogenous expression of self-renewal-stimulating factor genes in mESCs was specifically altered according to type of extracellular signaling. In the mESCs experiencing Stat3 signaling activated by LIF, 3D culture caused significant increase of the expression of all self-renewal-stimulating factor genes and integrin stimulation inside 3D culture induced significant increase of only *Bmp4* expression. Whereas, in the absence of Stat3 signaling, transcriptional up-regulation resulted from 3D culture of mESCs was only detected in *Wnt3a*, and integrin stimulation inside 3D culture significantly stimulated transcriptional level of *Lif* and *Bmp4* and significantly inhibited transcriptional level of *Wnt3a*. Moreover, under each extracellular signaling condition, Stat3 signaling itself stimulated or inhibited expression of specific self-renewal-stimulating factor genes, with inducing no alte-

ration. Entirely, regardless of Stat3 signaling by LIF, integrin-stimulating 3D culture of mESCs induced no transcriptional down-regulation of self-renewal-stimulating factor genes (excluding *Wnt3a* in the absence of LIF), indicating that integrin signaling inside 3D culture may effectively support mESC self-renewal through induction of endogenous secretion of self-renewal-stimulating factors without supplementation of any self-renewal-stimulating factors.

In detail, our study showed significant high expression level of *Lif* and *Bmp4* in integrin-stimulating 3D culture system relative to 3D culture system in the absence of exogenous LIF and such expression levels were not different with that in the presence of exogenous LIF. These results suggest that integrin stimulation of mESCs within 3D culture system can both contribute to express endogenous *Lif* and *Bmp4* and replace the role of exogenous LIF at least within the framework of *Lif* and *Bmp4* expression, although broad spectrum of the related gene expression remains to be evaluated. In addition, the data showed that *Wna3a* expression of mESCs was decreased when the exogenous LIF signal was removed in integrin-stimulating 3D culture system, indicating that integrin stimulation has the role to inhibit *Wnt3a* expression under 3D culture system and such inhibitory role is blocked by exogenous LIF. It has well-known that LIF and BMP4 are main factors to maintain pluripotency of mESCs (Smith *et al*., 1988; Williams *et al*., 1988; Qi *et al*., 2004) and both molecules cooperate to achieve such aim as main factors (Ruzinova and Benezra, 2003; Ying *et al*., 2003). In addition, Wnt3a is also an important factor in mESCs because it plays a role to support self-renewal of mESCs (Singla *et al*., 2006). These main factors as well as other growth factors for the pluripotency of mESCs can be usually provided from serum and feeder cells, exogenously, and/or endogenous expression by mESCs themselves. Of those, to induce endogenous expression of all main factors for mESC pluripotency by simple stimulation is going to be the most ideal method for supplying various factors and it will be able to provide an ease of cellular analysis by simplifying exogenous stimulant as well as provide cost-benefit for culturing mESCs. In this viewpoint, our results may suggest a possibility for establishment of simple system to endogenously induce important factors such as LIF and BMP4 for mESCs maintenance by adjusting extracellular signaling under 3D system. Furthermore, the negative regulation of *Wnt3a* expression in mESCs by integrin signaling under 3D system suggests that additional efforts should be made to develop an optimized system to be able to remedy current limitation.

In conclusion, appropriate regulation of extracellular signaling through alteration of culture dimension and activation of active integrin heterodimers and self-renewal-stimulating intracellular signaling will be able to provide a feasibility to exactly regulate the maintenance, growth, and differentiation of mESCs, and additional studies about another extracellular signaling stimulating endogenous secretion of self-renewal-stimulating factors should be conducted. Subsequently, comprising the synthetic 3D niche to increase endogenous level of self-renewal-stimulating factors will provide a cost-effective system for mESCs culture.

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