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Effects of Extracellular Stimulation of Different Niche Condition on the Transcriptional Regulation of Matrix Metalloproteinase Genes in the Mouse Embryonic Stem Cells

Jung Im Yun[1], Min Seong Kim[2] and Seung Tae Lee[2,†]

[1]*Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Korea*
[2]*Department of Animal Biotechnology, Kangwon National University, Chuncheon 200-701, Korea*

ABSTRACT

Matrix metalloproteinases (MMPs) have been known to affect to cell migration, proliferation, morphogenesis and apoptosis by degrading the extracellular matrix. In the previous studies, undifferentiated mouse embryonic stem cells (ESCs) were successfully proliferated inside the extracellular matrix (ECM) analog-conjugated three-dimensional (3D) poly ethylene glycol (PEG)-based hydrogel. However, there is no report about MMP secretion in ESCs, which makes it difficult to understand and explain how ESCs enlarge space and proliferate inside 3D PEG-based hydrogel constructed by crosslinkers containing MMP-specific cleavage peptide sequence. Therefore, we investigated what types of MMPs are released from undifferentiated ESCs and how extracellular signals derived from various niche conditions affect MMP expression of ESCs at the transcriptional level. Results showed that undifferentiated ESCs expressed specifically *MMP*2 and MMP3 mRNAs. Transcriptional up-regulation of *MMP*2 was caused by the 3D scaffold, and activation of integrin inside the 3D scaffold upregulated *MMP*2 mRNAs synergistically. Moreover, mouse embryonic fibroblasts (MEFs) on 2D matrix and 3D scaffold induced upregulation of *MMP3* mRNAs, and activation of integrins through conjugation of extracellular matrix (ECM) analogs with 3D scaffold upregulated *MMP3* mRNAs synergistically. These results suggest that successful proliferation of ESCs inside the 3D PEG-based hydrogel may be caused by increase of MMP2 and MMP3 expression resulting from 3D scaffold itself as well as activation of integrins inside the 3D PEG-based scaffold.

(Key words : Matrix metalloproteinase, 3D, Polyethylene glycol, Extracellular signaling, Mouse embryonic stem cells)

INTRODUCTION

Matrix metalloproteinases (MMPs) have reported to constitute a family of more than 25 enzymes and their functionalities are dependent on the zinc ion at the active site, the cysteine switch motif in the propeptide and the zinc-binding domain in the catalytic domain (Vise *et al.*, 2003; Nagase *et al.*, 2006; Cauwe *et al.*, 2007). Generally, MMPs play an important role in remodeling tissues by degrading extracellular matrix consisting of collagen, laminin, fibronectin, entactin/nidogen and heparin sulphate proteoglycan. Therefore, a variety of physiological events, such as cell migration, proliferation, morphogenesis and apoptosis, are caused by extracellular matrix (ECM) breakdown and modification resulted from MMP activity (Vu *et al.*, 2000; Mannello *et al.*, 2005; Page-McCaw *et al.*, 2007).

In the previous studies (Lee *et al.*, 2010; Lee *et al.*, 2012; Jang *et al.*, 2013), we observed that mouse embryonic stem cells (ESCs) could cleave MMP-specific cleavage peptide sequence, which resulted in proliferation of ESCs inside 3 dimensional (D) polyethylene glycol (PEG)-based hydrogel polymerized by crosslinkers with MMP-specific cleavage site. Moreover, ESCs experiencing extracellular signaling like integrin activation inside 3D PEG-based hydrogel showed significantly stronger proliferative ability of ESCs. However, the absence of reports about MMP effects on maintenance of stem cell undifferentiation made it difficult to understand and explain the phenomenon. Accordingly, we investigated type and transcriptional level of MMP genes expressed in undifferentiated ESCs and the effects of different culture dimension and extracellular signals under 2D or 3

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† Corresponding author : Phone: +82-33-250-8638, E-mail: stlee76@kangwon.ac.kr

D condition on the transcriptional regulation of MMP genes in undifferentiated ESCs.

MATERIALS AND METHODS

ESC Culture

The mouse ESC E14tg2a was purchased from ATCC (Manassas, VA) and cultured on 10 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, MO)-treated mouse embryonic fibroblasts (MEFs) in 5% CO_2 and 37℃ in standard ESC culture medium. 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 leukemia inhibitory factor (LIF; Chemicon International, Temecula, CA) was used as standard ESC culture medium. Moreover, subpassage of confluent ESCs was conducted every third day and medium was changed every day during subculture.

Encapsulation of ESCs into PEG-based Hydrogel and Culture

According to previously described protocols (Lee *et al.*, 2010), PEG-based scaffold was constructed, cell adhesion peptides were conjugated into the 3D PEG-based scaffold and ESCs were incorporated into the 3D PEG-based scaffold. Subsequently, ESCs inserted within a 10% (w/v) PEG-based scaffold without or with adhesion peptides were cultured in standard ESC culture medium. After culture for 5 days, retrieval of ESCs from the PEG-based scaffold was conducted by incubating in 10 mg/ml collagenase I (Sigma-Aldrich) solution for 10 minutes.

Extraction of mRNA and Synthesis of cDNA

According to manufacturer's instruction, the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) was used for extraction of total mRNA from ESCs and synthesis of c-DNA from extracted mRNA was conducted using a Reverse Transcription System (Promega, Madison, WI).

Real-Time PCR

Designation of all the oligonucleotide primer sequences shown in Table 1 was performed with cDNA sequence obtained from GenBank for mouse and by Primer3 software (Whitehead Institute/MIT Center for Genome Research). The Bio-rad iCycler iQ system (Bio-Rad Laboratories, Hercules, CA) was used for PCR amplification and quantification of gene expression was performed using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories). Melting curve data was collected for identifying PCR specificity and the specific gene expression was normalized by comparison to the *β-actin* transcrip_tional level. Relative mRNA level was presented as $2^{-\Delta\Delta Ct}$, where Ct=threshold cycle for target amplification, $\Delta Ct=Ct_{target\ gene}$ (specific genes for each sample)$-Ct_{internal\ reference}$ (β-actin for each sample), and $\Delta\Delta Ct=\Delta Ct_{sample}$ (treatment sample in each experiement)-$\Delta Ct_{calibrator}$ (control sample in each experiment).

Statistical Analysis

The Statistical Analysis System (SAS) program was used for the statistical analysis of all the numerical data shown in each experiment. When a significance of the main effects were detected by analysis of variance (ANOVA) analysis in the SAS package, the least-square or DUNCAN methods were performed to compare among treatments. Moreover, p values less than 0.05 were regarded as indicative of significant differences.

Table 1. Oligonucleotide primers and PCR cycling conditions

Genes	GenBank number	Primer sequence		Size (bp)	Temp (℃)
		Sense (5'>3')	Anti-sense (5'>3')		
β-actin	X03672	TACCACAGGCATTGTGATGG	TCTTTGATGTCACGCACGATT	200	60
MMP-2	NM_008610	CCATCGAGACCATGCGGAAG	AAGGCCCGAGCAAAAGCATC	175	60
MMP-3	NM_010809	TGGCCACTCCCTGGGACTCT	CATCAGGGGATGCTGTGGGA	161	60
MMP-7	NM_010810	CCTTTGATGGGCCAGGGAAC	CCAAATTCATGGGTGGCAGC	154	60
MMP-8	NM_008611	TGCCACGATGGTTGCAGAGA	CCACTTGGGACTTCCTGGGG	166	60
MMP-9	NM_013599	AGTTGTGGTCGCTGGGCAAA	CTACACCAAGGCGTGCCGTC	154	60

RESULTS

Identification of MMP Genes Expressed in Undifferentiated Mouse ESCs

In order to identify which type of MMP genes were expressed in undifferentiated ESCs, transcriptional level of MMP genes were measured in ESCs conventionally cultured on 2D MEFs in standard ESC culture medium. As shown in Fig. 2, among total 5 MMP genes, transcription of *MMP2* and *MMP3* were justly detected, whereas no transcription was observed in *MMP7*, *MMP8* and *MMP9*. Moreover, compared to *MMP3* transcriptional level, *MMP2* showed significantly approximately 5 times higher transcriptional level. From these results, we propose that mouse ESCs may secrete specifically MMP2 and MMP3 during maintenance of undifferentiated state.

The Effects of Extracellular Signaling Derived from Different Niche on the Transcriptional Regulation of *MMP2* and *MMP3* Expressed in Undifferentiated Mouse ESCs

Subsequently, for investigating how transcription of *MMP2* and *MMP3* in undifferentiated ESCs is regulated by extracellular environment components, ESCs were cultured on 2D MEFs-free ('2D'; Fig. 1B) or -containing culture plate ('MEFs'; Fig. 1A) and inside 3D PEG-based hydrogel without ('3D'; Fig. 1C) or with ('Activation of Integrins'; Fig. 1D) ECM analogs activating integrin $\alpha_5\beta_1$, $\alpha_V\beta_5$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$ expressed actively in the undifferentiated ESCs (Lee *et al.*, 2010). Then, transcriptional level of *MMP2* and *MMP3* was measured and compared among each experiment group.

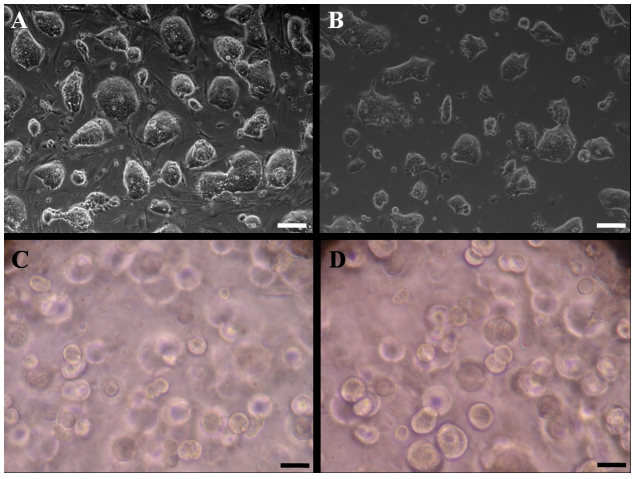

Fig. 1. Morphology of mouse embryonic stem cells (ESCs) cultured for 3 days on mouse embryonic fibroblasts (MEFs)-free or -containing 2D culture plate and for 5 days within the 3D polyethylene glycol (PEG)-based scaffold without or with activation of integrin $\alpha_5\beta_1$, $\alpha_V\beta_5$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$. ESCs were cultured in standard ESC culture medium and integrin $\alpha_5\beta_1$ and $\alpha_V\beta_5$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$ were activated by incorporating ESCs into PEG-based scaffold conjugated with 400 μM RGDSP, 800 μM TTSWSQ, and 800 μM AEIDGIEL. The ESCs cultured on 2D MEFs showed uniformly concentric and compact colonies, and spreading and flattened colonies were partially observed in the ESCs cultured on the MEFs-free 2D matrix. However, the ESCs cultured inside the 3D PEG-based scaffold showed spherical morphology, regardless of integrins stimulation. The scale bars are 100 μm.

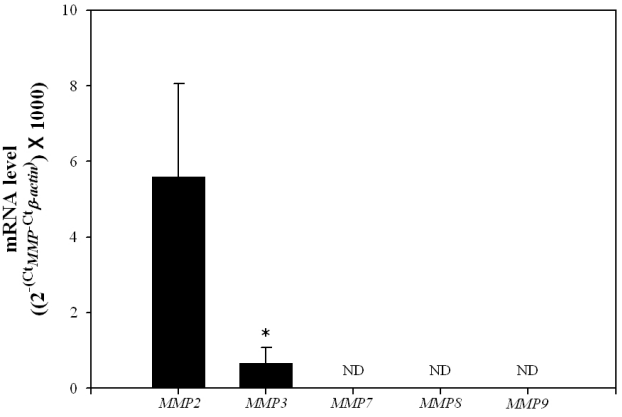

Fig. 2. Transcriptional regulation of matrix metalloproteinase (MMP) in undifferentiated mouse embryonic stem cells (ESCs). Among total 5 MMP genes, transcription of *MMP2* and *MMP3* were detected in undifferentiated ESCs through real-time PCR and transcriptional level of *MMP2* were significantly higher than those of *MMP3*. Whereas, no transcriptional regulation of *MMP7*, *MMP8* and *MMP9* was identified in undifferentiated ESCs. All data shown are mean±S.D. of five independent experiments. $^*p<0.05$. ND= not detected.

In case of *MMP2* expression (Fig. 3), ESCs experiencing integrin activation inside 3D PEG-based hydrogel conjugated with ECM analogs showed significantly the highest transcriptional level (approximately 34 times transcriptional up-regulation compared to '2D Matrix' group and approximately 5.8 times transcriptional up- regulation compared to '2D MEFs Matrix' group) and extracellular stimulation derived from 2D MEFs-containing culture plate induced significant increase of *MMP2* expression (approximately 5.9 times transcriptional up-regulation), compared to '2D Matrix' and '3D Scaffold' groups. Whereas, no significant difference in the expression of *MMP2* was detected in ESCs cultured between on 2D matrix and in 3D scaffold condition.

Transcriptional level of *MMP3* (Fig. 4) was significantly the highest in 'Activation of Integrins inside 3D Scaffold' group (approximately 1280 times transcriptional up-regulation compared to '2D Matrix' group and approximately 10.1 to 11.5 times transcriptional up-regulation compared to '2D MEFs Matrix' and '3D Sca-

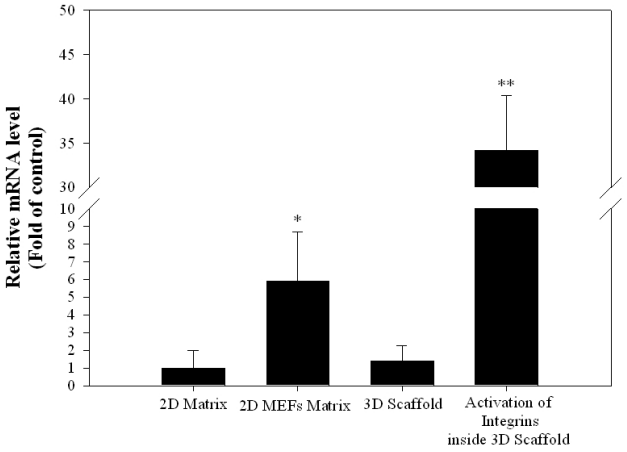

Fig. 3. Effects of extracellular signaling derived from different niche condition on the transcriptional regulation of *MMP2* in mouse embryonic stem cells (ESCs). The ESCs were cultured in mouse embryonic fibroblasts (MEFs)-free ('2D Matrix') or -containing ('2 D MEFs Matirx') culture plate with subpassage every 3 day and inside 3D polyethylene glycol (PEG)-based scaffold conjugated with ('Activation of Integrins inside 3D Scaffold') or without ('3D Scaffold') 400 μM RGDSP, 800 μM TTSWSQ, and 800 μM AEIDGIEL for integrin $\alpha_5\beta_1$ and $\alpha_V\beta_5$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$ for 5 days without subpassage. Subsequently, transcriptional level of *MMP2* was analyzed by real-time PCR. Entirely, significant transcriptional up-regulation of *MMP2* was induced by extracellular signaling in each culture dimension and dimensional difference did not cause any alteration in transcriptional regulation of *MMP2*. Moreover, activation of integrins inside 3D scaffold showed significantly higher transcriptional level of *MMP2* than extracellular signal derived from MEFs on 2D matrix. All data shown are mean ±S.D. of five independent experiments. $^{*,**}p<0.05$.

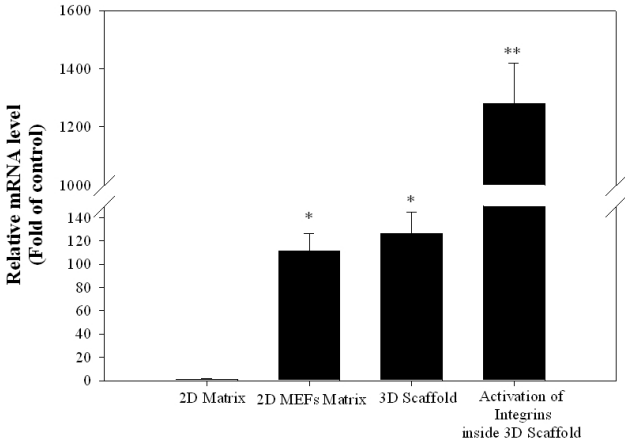

Fig. 4. Effects of extracellular signaling derived from different niche condition on the transcriptional regulation of *MMP3* in mouse embryonic stem cells (ESCs). The ESCs were cultured in mouse embryonic fibroblasts (MEFs)-free ('2D Matrix') or -containing ('2D MEFs Matrix') culture plate with subpassage every 3 day and inside 3D polyethylene glycol (PEG)-based scaffold conjugated with ('Activation of Integrins inside 3D Scaffold') or without ('3D Scaffold') 400 μM RGDSP, 800 μM TTSWSQ, and 800 μM AEIDGIEL for integrin $\alpha_5\beta_1$ and $\alpha_V\beta_5$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$ for 5 days without subpassage. Subsequently, transcriptional level of *MMP3* was analyzed by real-time PCR. Significantly the highest transcriptional level of *MMP3* was observed in ESCs experiencing activation of integrin inside 3D scaffold. Moreover, extracellular signals derived from 3D scaffold itself ('3D Scaffold') and MEFs on 2D matrix ('2D MEFs Matrix') showed significant increase in the transcriptional level of *MMP3*, compared to MEFs-free 2D matrix ('2D Matrix'), and no significant difference was detected between '3D Scaffold' and '2D MEFs Matrix' group. All data shown are mean±S.D. of five independent experiments. $^{*,**}p<0.05$.

ffold' group) and significantly the lowest transcriptional level of *MMP3* was detected in ESCs cultured on 2D MEFs-free culture plate. However, extracellular signaling derived from MEFs on 2D matrix (approximately 111 times transcriptional up-regulation) and 3D scaffold (approximately 126 times transcriptional up-regulation) induced significant increase in the transcriptional level of *MMP3*, compared to '2D Matrix' group and ESCs cultured between on 2D MEFs matrix and in 3D scaffold condition showed no significant difference in the *MMP3* transcriptional regulation. These results demonstrate that extracellular signaling derived from different niche, such as 2D matrix, MEFs on 2D matrix, 3D scaffold and activation of integrin inside 3D scaffold, play an important role in regulating transcription of MMP genes in undifferentiated ESCs.

DISCUSSION

In this study, the presence of extracellular signaling in the same culture dimension increased transcriptional level of all MMP genes (*MMP2* and *MMP3*) expressed in undifferentiated ESCs and activation of all integrin heterodimers expressed in undifferentiated ESCs inside 3D scaffold greatly up-regulated transcriptionally all MMP genes expressed in undifferentiated ESCs. Accordingly, we could identify that signaling derived from extracellular microenvironment components enclosing undifferentiated ESCs played a very major role in regulating type and transcriptional level of transcribed MMP genes.

The highest increase of *MMP2* and *MMP*3 expression in ESCs cultured inside 3D PEG-based hydrogel conjugated with ECM analogs stimulating integrin $\alpha_5\beta_1$, $\alpha_V\beta_5$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$ may result in increasing greatly active MMP secretion, which will be able to make it easy to enlarge proliferative space of ESCs inside 3D scaffold and to proliferate actively ESCs inside 3D scaffold as previously described (Lee *et al.*, 2010; Lee *et al.*, 2012; Jang *et al.*, 2013). Moreover, transcription of *MMP2* and *MMP3* and no transcription of *MMP7*, *MMP8* and *MMP9* identified in undifferentiated ESCs may be able

to be used as ESC-specific maker genes through comparison of MMP expression patterns in differentiated ESCs.

In conclusion, we could identify indirectly that improved secretion of MMP2 and MMP3 resulted from 3D scaffold and integrin activation inside 3D scaffold made it possible to proliferate successfully ESCs inside 3D PEG-hydrogel polymerized by MMP-specific cleavage site-containing crosslinkers by identifying significant increase of MMP2 and MMP3 transcriptional level. Moreover, because MMPs are closely related with tissue remodeling during embryogenesis (Hillegass *et al.*, 2007), studies on the relevance of MMP in ESC differentiation should be requested further.

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