DISTINCT AUTO-REGULATION OF EMBRYONIC STEM CELL BEHAVIOR BY CELL-SECRETED SOLUBLE FACTORS IN A MEMBRANE-BASED TWO-CHAMBERED MICROBIOREACTOR

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ABSTRACT
We report undifferentiated culture or differentiation of mouse embryonic stem cells (mESCs) in a membrane-based two-chambered microbioreactor (MB) by maintaining small volume in the top chamber while providing enough volume in the bottom chamber to maintain nutrient level. We observed that upregulated BMP4 in the MB regulated both the undifferentiation and differentiation behaviors differently as compared with the 6-well plate (6WP) cultures where BMP4 was downregulated. In addition, by utilizing mathematical model of the undifferentiated cultures, we showed that volume of the MB was suitable to activate BMP4 signaling whereas that of the 6WP was not.

KEYWORD
Microbioreactor, cell-secreted soluble factors, embryonic stem cells, pluripotency, differentiation, mathematical modeling.

INTRODUCTION
Soluble factors in the microenvironment play an important role on ESC undifferentiation and differentiation behaviors. For establishing an *in vitro* differentiation system, it is essential to consider influence of exogenously added soluble factors as well as cell-secreted ones (i.e. endogenous soluble factors) on cell behavior [1].

In enclosed micro-scale static culture systems, cell-secreted soluble factors can accumulate in a small volume as well as remain in the cell neighborhood for long-time because of the diffusion dominant nature of the microenvironment [2]. These capabilities of the micro-scale culture systems provide opportunity to study influence of cell-secreted soluble factors on cell behavior in an *in vivo* relevant dimension and manner. However, previous microfabrication based studies primarily focused on flow and size (diameter of ESC aggregates i.e. EBs) dependent modulation of soluble microenvironment to direct ESC behavior [3-6]. No study focused on the influence of cell-secreted factors in a small culture volume, which might strongly influence ESC behavior. Characterization of this influence is important for developing robust culture systems to control ESC behavior through a better understanding of soluble factor signaling.

In this context, we were interested to study ESC behavior in an *in vivo* mimicking microenvironment which can realize accumulation and retention of the cell-secreted factors around the cells. In fact, pluripotent stem cells are enclosed by trophectoderm and extra-embryonic part *in vivo*. Early fate change of the stem cells occurs in a diffusion dominant microenvironment of the enclosure while nutrient supply to the enclosed cells is provided by maternal side. Therefore, to culture and differentiate mESC in an in vivo mimicking microenvironment, we are interested in utilizing a membrane-based two-chambered microbioreactor (MB).

EXPERIMENT
In the MB, mESCs were cultured (undifferentiated culture) and differentiated on a polyester membrane (culture area 2.27 cm^2) sandwiched between two chambers made of PDMS (Fig. 1A). Height and volume of each chamber were 500 μm and 114 μl, respectively. During the culture, we maintained small culture volume in the top chamber to form the diffusion dominant microenvironment (Fig. 1A) while culture volume in the bottom chamber was adjusted to provide enough nutrient supply for static culture. In the MB, we maintained undifferentiated culture of mESCs for 5 days, and differentiated mESCs for 8 days by using chemically defined medium with and without leukemia inhibitory factor (LIF), respectively. To determine the undifferentiated or differentiated state of mESCs, we evaluated relative expression of various genes by qPCR. To elucidate role of cell-secreted soluble factors, we did inhibition experiments in the cultures.

To understand how physical aspects of a culture system can influence cell-secreted soluble factor signaling, we developed mathematical models of the undifferentiated cultures of mESCs in the MB and 6WP by using Comsol Multiphysics 4.1 software program (Comsol, Inc.). The models quantified autocrine activity of BMP4 (proportional to concentration of BMP4-bound receptors) in the culture systems by considering diffusion of BMP4 in culture volume; diffusion and secretion of BMP4, binding of BMP4 to its receptor, etc. in mESC aggregates.

RESULTS AND DISCUSSION
In both undifferentiated and differentiated cultures, mESC and differentiated cell morphology differed markedly between the MB and 6WP cultures (Fig. 1B). In the undifferentiated culture, mESCs maintained dome-shaped colonies in the MB, whereas colonies were extensively merged in the 6WP (Fig. 1B). In case of the differentiated cultures, cells formed epithelial sheet-like morphology in the MB, whereas cells in the 6WP formed aggregate having neuronal extensions (Fig. 1B).

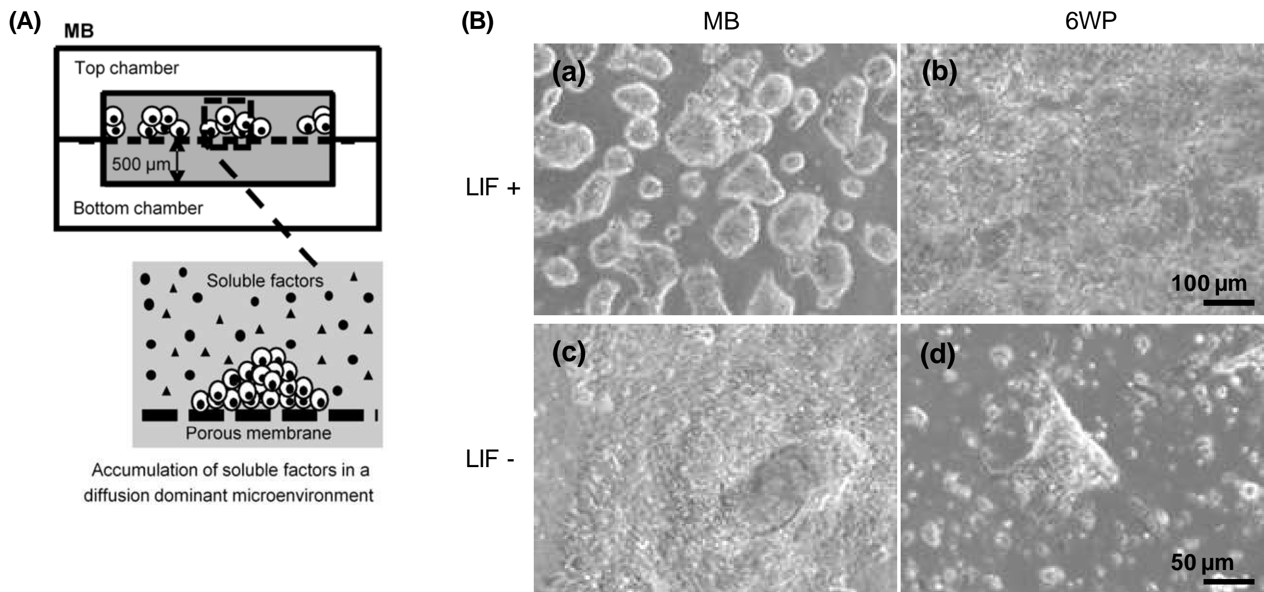

Figure 1. (A) Schematic showing cross-section of the membrane-based two-chambered microbioreactor (MB). (B) Morphology of undifferentiated mESCs (LIF+ culture on day5; (a) and (b)) and differentiated cells (LIF- culture on day 8; (c) and (d)) in the MB ((a) and (c)) and 6WP ((b) and (d)).

Comparison of gene expression between the MB and 6WP revealed that mESCs maintained a high pluripotent state (Fig. 2a) and differentiated towards meso- and endo-derm (Fig. 2b) in the undifferentiated and differentiated cultures in the MB, respectively. In case of cell-secreted soluble factors, expression of BMP4 was remarkably upregulated in the undifferentiated and differentiated cultures in the MB than the 6WP (Fig. 2).

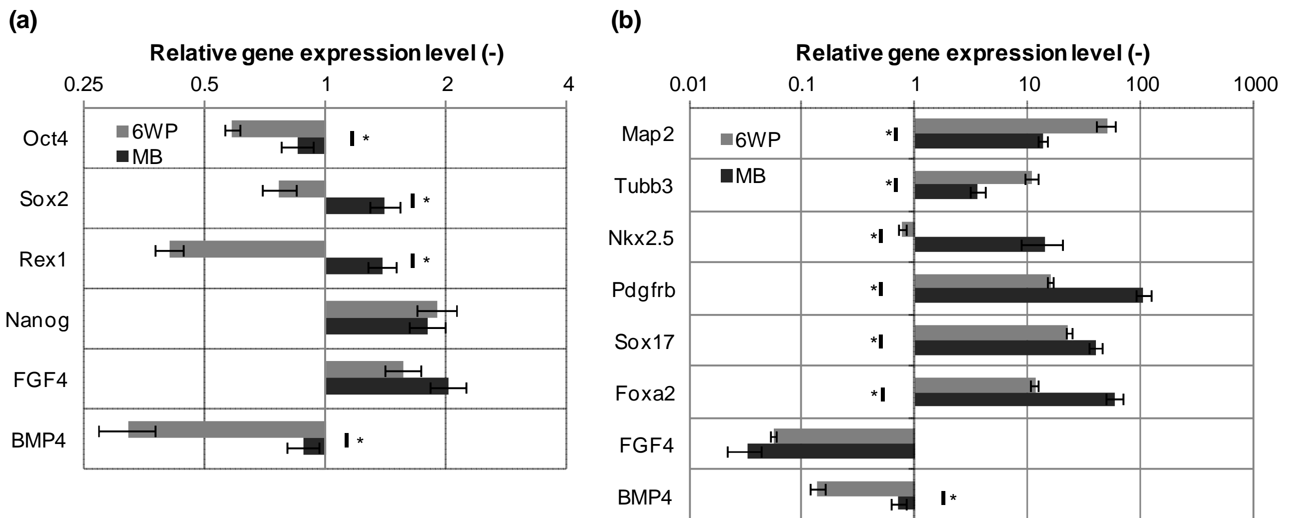

Figure 2. (a) Expression of pluripotency markers (Oct4, Sox2, Rex1 and Nanog) and cell-secreted soluble factors (FGF4 and BMP4) in mESCs cultured for 5 days in the MB and 6WP with LIF. (b) Expression of neuronal markers (Map2, Tubb3), mesoderm markers (Nkx2.5, Flk1), endodermal markers (Sox17, Foxa2) and cell-secreted soluble factors (FGF4, BMP4) in differentiated cells on day 8 in the MB and 6WP.Expression level one represents gene expression in the undifferentiated mESCs cultured for 2 or 3 days. Statically significant difference is indicated by the symbol "".*

Inhibition of BMP4 signaling by its antagonist Noggin caused downregulation of pluripotency markers in the undifferentiated culture (Fig. 3b) and meso- and endo-derm markers in the differentiated culture in the MB (Fig. 3b). In contrast, in the 6WP, BMP4 was not upregulated (Fig. 2) and had negligible effect on mESC behaviors (data not shown). Therefore, cell-secreted BMP4 controlled mESC behaviors in the MB but not in the 6WP.

By using mathematical model of undifferentiated culture in the MB or 6WP, we determined the time (whole culture period) and volume averaged BMP4-bound receptor concentration to represent the signaling activity in a mESC colony at various values of culture volume height. The signaling activity changed as an inverse function of height (Fig. 4). When the culture medium height was ≥ 1 mm, signaling activity changed little with the variation of height (Fig. 4). However,

the signaling activity increased sharply when the height was below 1 mm (Fig. 4). The height of the culture medium in the 6WP (2 mm) was in the flat region of the curve (Fig. 4). In contrast, the height (each chamber height = 0.5 mm) of the MB was in the sharply increasing region of the curve. Therefore, the size of the MB, but not of the 6WP, was associated with upregulated BMP4 signaling, as observed in the experimental results.

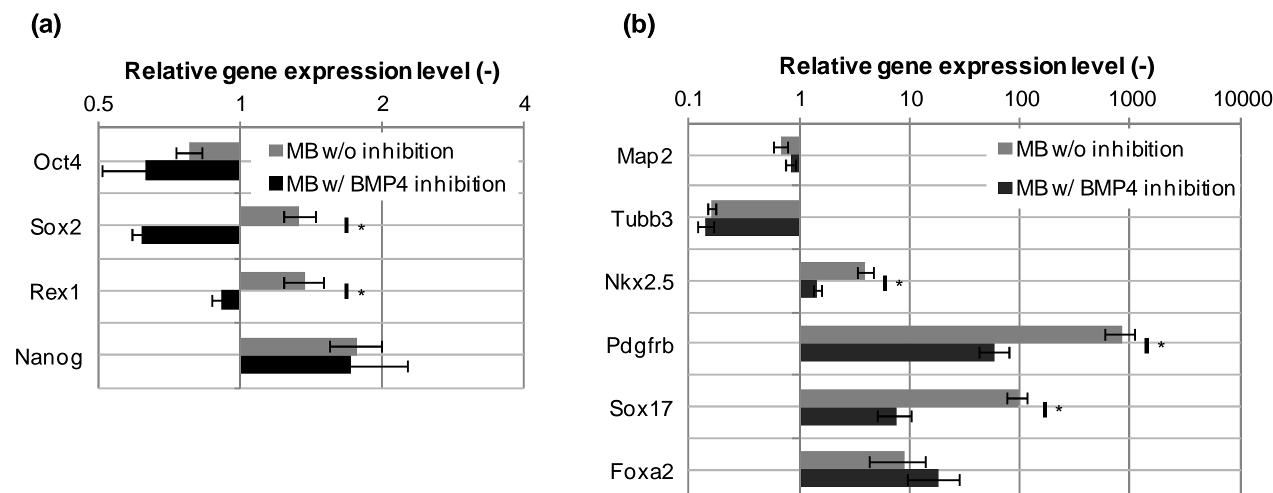

Figure 3. (a) Expression of pluripotency markers (Oct4, Sox2, Rex1 and Nanog) in undifferentiated mESC cultured for 5 days and (b) Expression of neuronal markers (Map2, Tubb3), mesoderm markers (Nkx2.5, Flk1), endodermal markers (Sox17, Foxa2) in differentiated cells on day 8 in the MB with and without the inhibition of BMP4 by its antagonist Noggin. Expression level one represents gene expression in the undifferentiated mESCs cultured for 2 or 3 days. Statically significant difference is indicated by the symbol "".*

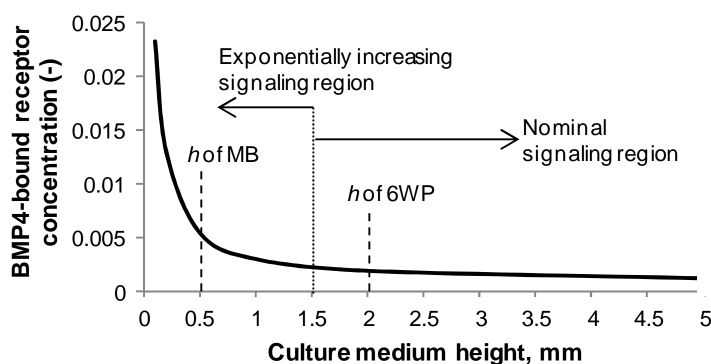

Figure 4. Variation of the time and volume averaged dimensionless BMP4-bound receptor concentration in a colony based on the variation of culture medium height (h). The culture medium height in a single chamber of the MB and 6WP is marked by vertical dashed lines.Dotted-line separated two distinct region of signaling as a function of height .

CONCLUSION

In this study, for the first time, we showed that a small-scale membrane-based two-chambered bioreactor (MB) facilitated enriched soluble factor signaling environment than the conventional large-scale culture systems. Moreover, owing to the membrane-based design, long-term culture in the MB was possible by maintaining small culture volume in the top chamber while maintaining enough culture volume for nutrient in the bottom chamber. This is not possible in a single-chambered microbioreactor thereby limiting their use in long-term culture study. By mathematical modeling, we also estimated appropriate range of height of the culture medium in a culture system to activate cell-secreted soluble factor signaling. Therefore, this study will be helpful for studying EB differentiation, other stem cell behavior and in designing advanced microfluidic cell culture systems.

REFERENCES

[1] M. V. Wiles and G. Proetzel, in Embryonic Stem cells A Practical Approach, ed. by E. Notarianni and M.J. Evans (Oxford University Press, New York, 2006), pp. 112–119.

[2] H. Yu, I. Meyvantsson, I. A. Shkel, and D. J. Beebe, Lab Chip **5**, 1089 (2005).

[3] E. Figallo, C. Cannizzaro, S. Gerecht, J. A. Burdick, R. Langer, N. Elvassore, and G. Vunjak-Novakovic, Lab Chip **7**, 710 (2007).

[4] W.-T. Fung, A. Beyzavi, P. Abgrall, N. -T. Nguyen, and H.-Y. Li, Lab Chip **9**, 2591 (2009).

[5] J. Park, C. H. Cho, N. Parashurama, Y. Li, F. Berthiaume, M. Toner, A. W. Tilles, and M. L. Yarmush, Lab Chip **7**, 1018 (2007).

[6] Y.-S. Hwang, B. G. Chung, D. Ortmann, N. Hattori, H.-C. Moeller, and A. Khademhosseini, Proc. Natl. Acad. Sci. U S A **106**, 16978 (2009).