論 文 の 内 容 の 要 旨

Membrane-based Two-chambered Microbioreactor for Controlling Embryonic Stem Cell Behavior

（膜構造を持つ２チャンバー型マイクロバイオリアクターを用いた胚性幹細胞の挙動制御）

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Abstract

Capabilities of embryonic stem cells (ESCs) to self-renew indefinitely and differentiate towards all types of mature functional cells hold great promises for regenerative medicine. To realize the promises, it is necessary to develop robust culture systems for controlling their capabilities. Autocrine/paracrine actions of cell-secreted soluble factors in the microenvironment play an important role on ESC undifferentiation and differentiation behaviors. Understanding the role and implementing the knowledge in the culture systems may provide better ways to control ESC behaviors. Although, microbioreactors are suitable for implementing cell-secreted soluble factor effects on cell behavior, there is no microbioreactor which properly realizes the implementation on ESC behaviors. In this thesis, a membrane-based two-chambered microbioreactor (MB) was developed to implement and understand cell-secreted soluble factor effects on ESC behavior in an *in vivo* mimicking microenvironment. *In vivo*, pluripotent stem cells in an embryo change their early fate in an enclosed diffusion dominant microenvironment while nutrient supply to the enclosed cells is provided by maternal side. To mimic the *in vivo* situation in the MB, I cultured or differentiated mESCs on the membrane in the top chamber which formed the diffusion dominant microenvironment**,** while the bottom chamber provided nutrient supply for the cells resembling the maternal side.

The top and bottom chambers of the MB were made from PDMS polymer by using techniques of microfluidics (photo-lithography and replica-molding). SiO_2 coating on the polyester membrane was necessary to bond the membrane with the chambers and the coating had negligible effects on glucose permeability of the membrane, growth and

expression of pluripotent markers in mESCs cultured on the membrane. Owing to Marangoni effect caused by surface tension differences at the open liquid-air interface, a soluble factor in an open macro-scale culture (e. g. 6-well plate/6WP) can be lost from cell neighborhood to bulk culture medium at a higher rate than that in an enclosed micro-scale culture (e.g. MB). To characterize how a soluble factor might disperse in the culture, I observed dye dispersion in the 6WP and MB in the absence of cells. Dyes of various molecular weights showed slower dispersion in the MB than that in the 6WP. Therefore, cells in the MB would be exposed to a higher concentration of cell-secreted soluble factors than in the macro-scale cultures owing to: 1) long-term retention of the factors around cell vicinity and 2) accumulation in the small volume. The higher concentration may activate autocrine signaling in the MB, thereby causing distinctive cellular response which may not be observed in the micro-scale cultures (e.g. 6WP).

Firstly, I cultured mESCs in the MB and investigated the effect of cell-secreted soluble factors on undifferentiated mESC behavior in the MB. After 5 days of culture, cell growth in the MB was similar to that in conventional 6-well plate (6WP) and membrane-based Transwell insert (TW) cultures, indicating adequate nutrient supply in the MB. However, the cells retained higher expression of pluripotency markers (Oct4, Sox2 and Rex1) and secreted soluble factors (FGF4 and BMP4) in the MB. Inhibition of FGF4 activity in the MB and TW resulted in a similar cellular response. However, inhibition of BMP4 activity revealed that – in contrast to the TW culture – autocrine action of the upregulated BMP4 cooperated with leukemia inhibitory factor (LIF) to upregulate the pluripotency markers expression in the MB culture. A higher concentration of BMP4 (because of higher accumulation and retention) than that in the 6WP or TW might have activated its positive feedback mechanism and upregulated its own expression in the MB.

Then, I investigated the comparative role of retention and accumulation in modulating BMP4 signaling in a macro- or micro-scale culture by utilizing models of mESC cultures in the MB and 6WP. These models quantified signaling activity of BMP4 by considering diffusion of BMP4 in culture volume; diffusion and secretion of BMP4, binding of BMP4 to its receptor, etc. in mESC aggregates. The models estimated that, owing to the small volume of the MB, concentration of BMP4 was higher in the MB culture than that in the 6WP. The higher BMP4 concentration induced

a higher concentration of BMP4-bound receptors in a mESC aggregate in the MB than that in the 6WP. The higher concentration of BMP4-bound receptors indicated enhanced activity of cell-secreted BMP4 signaling in the MB. By varying culture volume height (reflected the role of accumulation) and diffusion constant (i.e. diffusion rate/level of retention), I observed that variation in height would cause larger variation in the signaling activity in a culture than the variation in mass transfer rate would. Therefore, accumulation, rather than retention played crucial role in differing BMP4 signaling between the macro- and micro-scale cultures.

Secondly, I investigated early stage differentiation by differentiating mESCs in the MB for 8 days. Presence of extensive neuronal extensions from differentiated cell colonies indicated prominent ectoderm differentiation in the 6WP. However, in the MBs neuronal extensions were not visible and differentiated cells formed epithelial-sheet like morphology. Expression analysis of markers from three germ layers indicated higher differentiation of mESC towards ectoderm in the 6WP, whereas towards mesoderm and endoderm in the MBs. Furthermore, inhibition of BMP4 and FGF4 activity demonstrated that enhanced effect of upregulated BMP4 was responsible for the prominent mesoderm and endoderm differentiation in the MBs. However, in the 6WP, FGF4 directed the mESC differentiation towards ectoderm and downregulated BMP4 had a minimal influence on the differentiation behavior.

Finally, I present the conclusions and future outlook of the thesis. This study demonstrated utilization of microbioreactor to modulate cell-secreted soluble factor signaling by autoregulation and thereby enhancing or inducing alternative self-capabilities of ESCs. Understanding and implementation of the autoregulation of soluble factors similar to this study will lead to development of robust culture systems to control ESC or other stem cell (e.g. adult stem cell) behavior. Therefore, this study will contribute to chemical system engineering as well as biomedical and stem cell engineering and regenerative medicine approaches to modulate stem cell behavior for achieving the desired goal.