Laboratory of BioProcess Systems Engineering

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Laboratory of BioProcess Systems Engineering (BPSE) has been organized by Prof. Kino-oka since April 2009. The laboratory mission is to bring a good fortune in human life through the elucidation and utilization of "bio-potential" by understanding sequential biological events (BioProcess) in the reaction field (Systems). The main stream in BPSE, as shown in Fig.1, is the bioprocess designs in stem cell and tissue engineering. The research interests, which are targeted for the biological elements governing the reactions (*ie.* cellular differentiation, undifferentiation, de-undifferentiation, maturation, tissue self-assembling, functionalization etc.) as well as the regions providing the reaction field (*ie.* 2-D colonies and 3-D spherical and sheet aggregates), are to establish methodologies for process and quality controls in bio-production including the analysis, simulation, forecasting, making great contribution to the stem cell industry (*ie.* the outputs for manufacturing transplants through design of process and quality controls, for drug screening systems through development of bio-mimic structure, and for mass productions of human biologics through design of scale-up). The outcome includes the establishment of manufacturing process & quality controls (*ie.* culture design, bioreactor development, monitoring, process automation, etc.), the development of human resources as biochemical engineers, and establishment of standardizations and guidelines for stem cell industrialization.

BPSE were organized with four groups, (A) stem cell group for the maintenance of undifferentiation to prevent de-undifferentiation and for the directional regulation of differentiation, (B) cell expansion group for designs of culture process and intelligent

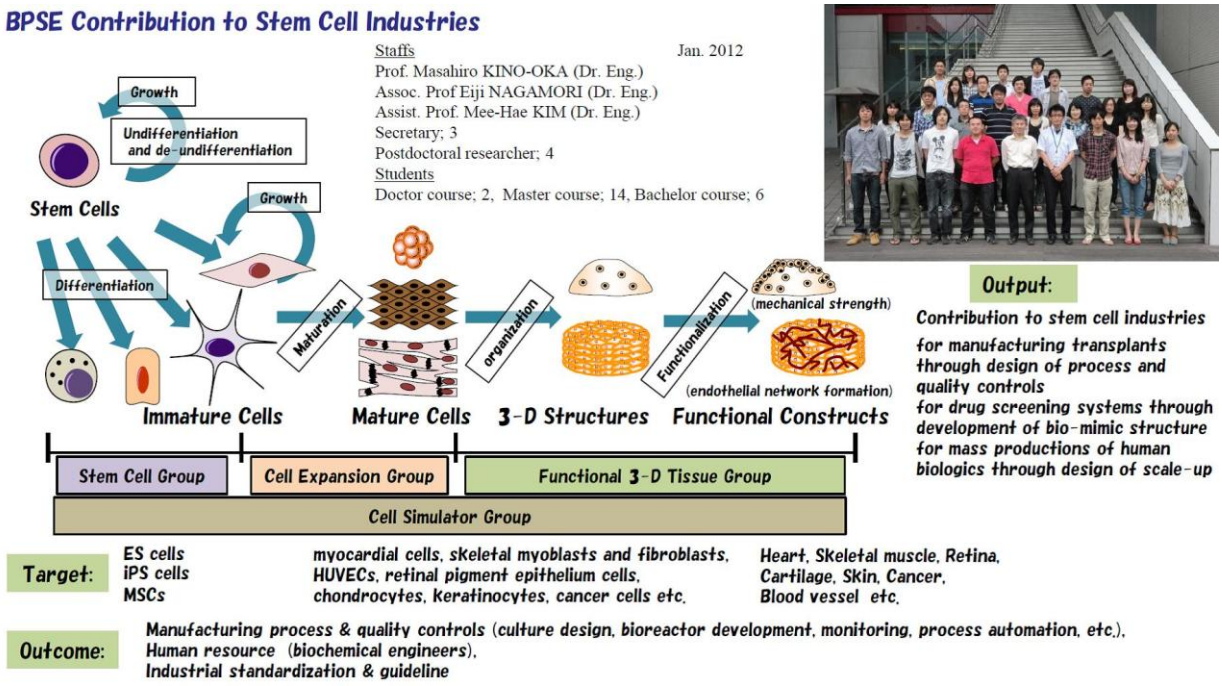

Fig.1 Mission of BPSE

automation system, (C) functional 3-D tissue group for vascular network formation a Local habitat distribution in multilayered cell sheet and mechanical strength in cultured tissue, and (D) culture simulation group for process and quality controls as an interdisciplinary technology supported by the above academic fields.

A. Stem Cell group

The main topics in this group are the methodological development for regulation of cytoskeletal signaling (Rho GTPase family of Rac1, cdc42, RhoA) by changing the cellular morphology with dynamic behaviors on the glucose-displaying dendrimer surface as shown in Fig.2.

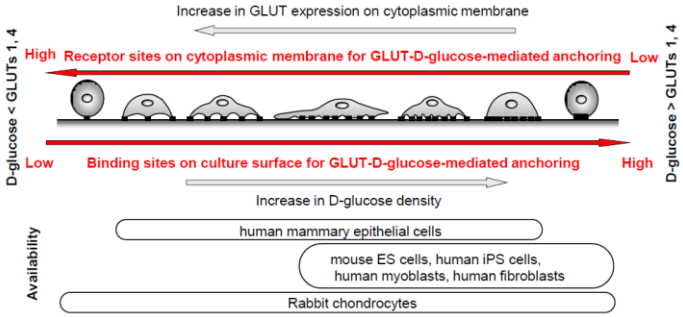

Fig.2 Availability of D-glucose displaying dendrimer surfaces

BPSE proposed a possible mechanism underlying the cell anchoring and morphological changes in cultures of human epithelial cells on D-glucose-displaying surfaces (1-8). The variation in the ratio of D-glucose displayed caused cellular morphological changes that depended on the presence or absence of insulin. In addition, fluorescence microscopy of F-actin, vinculin, and GLUTs clarified the localization of integrin-mediated as well as GLUT-mediated anchoring, leading to the consideration that the morphological changes of cells are responsible for the variation in the balance between the quantities of D-glucose on the culture surface and GLUTs on the cytoplasmic membrane, which is associated with the promotion of focal contact formation mediated by GLUTs. The review article (6) mentioned that the designs using the surfaces are based on the novel cell binding mechanism in which GLUTs are key elements responsible for regulating cell attachment and morphologies as well as development of the cellular phenotypes.

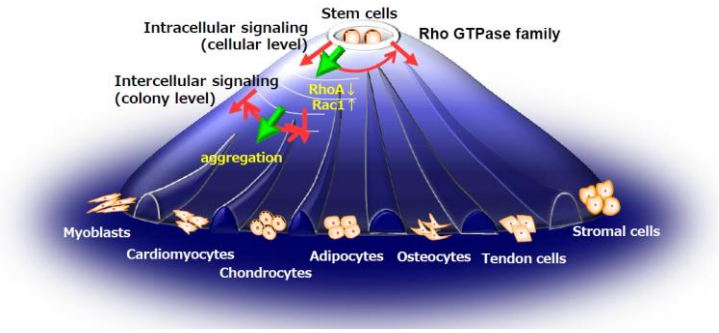

Fig.3 Directional regulation of stem cells based on the regulation of Rho GTPase family

Although specific ligand-receptor interactions on solid surfaces are important for regulating cell shapes in various cell types, the properties of the surfaces also play key roles in determining the cellular functions and fates. Identifying specific cues in microenvironment and understanding how neighboring cells and ECM control developmental fates will be required to promote the differentiation of cells, such as stem cells, into targeted cell lineages. BPSE proposed the idea for cytodifferentiation of stem cells accompanied by the regulation of cytoskeletal formation through cell morphological variation as well as the intracellular signal transduction through cell aggregate formation (Fig.3). BPSE recently reported that the alterations in Rho family GTPase activities derived from cytoskeletal formation can lead to

guidance of cardiomyogenic differentiation of human mesenchymal stem cells (hMSCs) during in vitro culture (8). To regulate the cytoskeletal formation of hMSCs, a dendrimer-immobilized substrate that displayed D-glucose was employed (Fig.4). With an increase in the dendrimer generation number, the cells exhibited active migration, accompanied by cell morphological changes of stretching and contracting without any modification of culture media. On the 5th-generation dendrimer surface, in particular, the cells exhibited RhoA down-regulation and Rac1 up-regulation during the culture, associated with alterations in the cellular morphology and migratory behaviors. It was also found that cell aggregate formation was promoted on this surface, supporting the notion that an increase in N-cadherin-mediated cell-cell contacts and Wnt pathway signaling regulate hMSC differentiation into cardiomyocyte-like cells. This suggests that the changes in the cytoskeletal organization have a direct impact on the cardiomyogenic differentiation of hMSCs in relation to Rho family GTPase-dependent signaling pathways. Thus, the transporter-mediating anchoring (D-glucose displaying on the surface) is one of the powerful techniques to realize the regulation of the cellular morphology accompanied by the cytoskeletal variation, leading to commitment of stem cells to different lineages. This technique currently applied to the maintenance culture of human iPS cells and can make the directional control to stay undifferentiated state of iPS cells without any derivation to de-undifferentiated state.

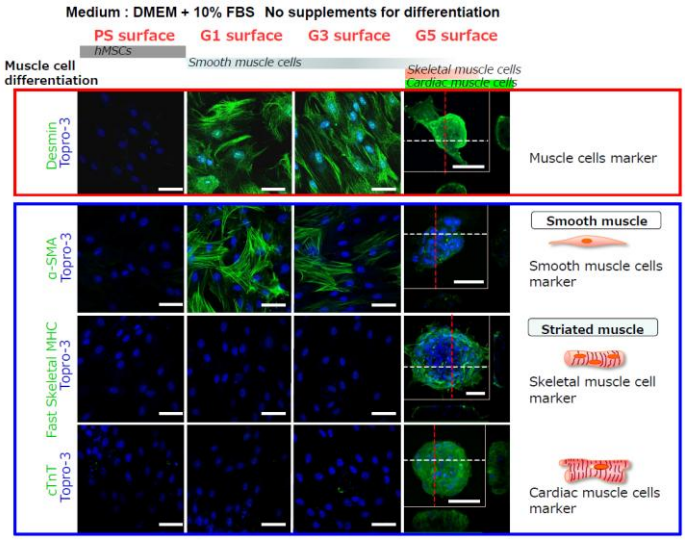

Fig.4 Cardiomyogenic differentiation capacities of hMSCs (7 days)

2. Cell Expansion group

Bioreactors are a core element to produce high-value materials in biological processes using animal cells. In the cultures of animal cells, the major features are the mechanical fragility and low growth rate of these cells, which leads to the strategy of high cell density culture by maintaining the cells within bioreactors with an external medium flow. In recent decades, technologies for cell and tissue therapies have emerged in the field of regenerative medicine. For such purposes, the targets are *ex vivo* expansion of human cells in undifferentiated or differentiated states, where the products of interest are cells and/or tissue themselves. This is an emerging interdisciplinary area of research and technology development that has the potential to revolutionize methods in medicine and leads to the requirement for small-scale design of patient-oriented bioreactors for clinical use. In addition, the bioreactor used in these cultures should be operated under strictly controlled conditions to produce cells or tissues with quality and functions that meet specific clinical requirements. BPSE has developed the automation for expansion process including the operations of seeding, medium change, passage as well as observation(9,10), and proposed the intelligent culture system accompanied by automated

operations (liquid transfer and cell passage) to perform serial cultures of human skeletal muscle myoblasts, as shown in Fig.5 (11). An automated culture system that could manage two serial cultures by monitoring the confluence degree was constructed. The automated operation with the intelligent determination of the time for passage was successfully performed without serious loss of growth activity, compared with manual operation using conventional flasks. This intelligent culture system can be applied to cultures of other adherent cells and will lead to the qualitative stability of products in the practical manufacturing of cells available for transplantation. In other words, the culture system will contribute to the process control as well as the quality control. Recently, this technique applied to the development of chip culture system for maturation of retina pigment epithelial cells derived from human iPS cells(Fig.6).

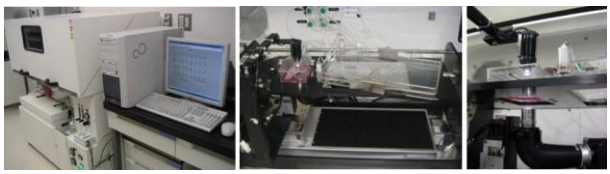

Fig.5 Intelligent bioreactor system for passage automation

Fig.6 Chip bioreactor system for maturation of retina pigment epithelial cells

Innovative techniques of cell and tissue processing, based on tissue engineering, have been developed for therapeutic applications. Cell expansion and tissue reconstruction through *ex vivo* cultures are core processes used to produce engineered tissues with sufficient structural integrity and functionality. In manufacturing, strict management against contamination and human error is compelled due to direct use of un-sterilable products and the laboriousness of culture operations, respectively. Therefore, the development of processing systems for cell and tissue cultures is one of the critical issues for ensuring a stable process and quality of therapeutic products. However, the siting criterion of culture systems to date has not been made clear. BPSE review article classifies some of the known processing systems into ‘sealed-chamber’ and ‘sealed-vessel’ culture systems based on the difference in their aseptic spaces, and describes the potential advantages of these systems and current states of culture systems (12). Moreover, on the basis of the guidelines for isolator systems used in aseptic processing for healthcare products, which are issued by the International Organization for Standardization, the siting criterion of the processing systems for cells and tissue cultures is discussed in perspective of manufacturing therapeutic products in consideration of the regulations according to the Good Manufacturing Practice.

On the basis of the siting criterion, the comparison of management between cell processing facility (CPF) and cell aseptic processing system (CAPS) based on the isolator system revealed that CAPS leads to reductions of the running cost as well as operational laboriousness in the small production. Recently, BPSE, as shown in Fig.7, proposed a novel design of manufacturing facility based on a flexible Modular Platform (fMP) which will reach the compactness of aseptic processing area and quick change-over for multi-purposes and patients, leading to cost-saving with safety and security .

As shown in Fig.8, BPSE collaborates with Profs. Okano and Shimizu in Tokyo Women's Medical University and several companies to develop the isolator system for the sheet assembly based on the fMP, which will make automated formation of multilayered sheets and their incubation. The machinery operations were successfully performed. And this system can realize some procedures by having flexible connections with various modules required for the culture operations under sterile conditions, suggesting the broad versatility for the production in other types of multilayered sheets.

Fig.8 Automation system of sheet assembly based on the fMP

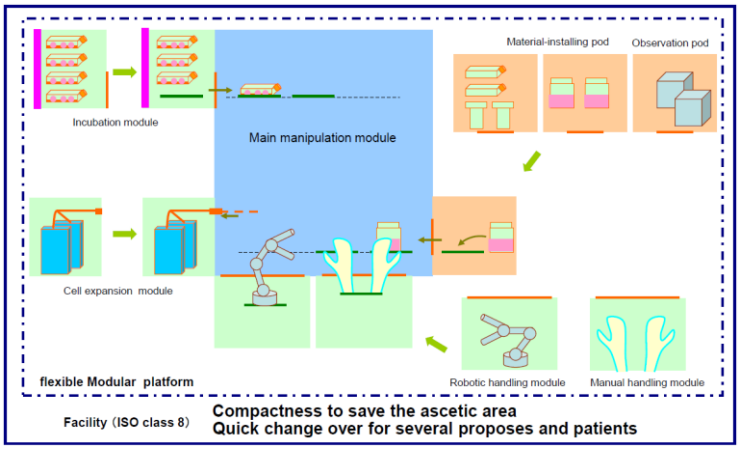

Fig.7 Proposal of manufacturing system based on flexible Modular Platform (fMP)

3. Functional 3-D tissue group

The innovative techniques based on tissue engineering have been developed, being now at a practical stage of clinical application by means of grafting in vitro cultured products. The representative process of autologous production of cultured tissues, which consists mainly of planar (monolayer) growth for cell expansion and spatial growth for tissue functioning. So far, a variety of natural and artificial materials available for scaffolds, which permit target cells to make aggregation, have been designed for the spatial growth and differentiation of the cells. The spatial growth in a scaffold leads to distribution in cell density owing to limitation of nutrient supply, causing the variation in quality of cultured tissues (spatial heterogeneity). In addition, another core process for cures using cells is the subculture for cell expansion to produce tissue-engineered tissues. However, the monolayer growth of target cells often causes the dedifferentiation accompanied with morphological variation (population heterogeneity), causing insufficient quality of cultured tissues. Therefore, it is necessary to evaluate the population heterogeneity as an indicator for state of differentiation during the subcultures, and to estimate spatial distribution of cell aggregate in cultured tissues. These techniques will be contributable to QC especially for efficacy of cultured tissues (13).

Many techniques for the QC were developed mainly for safety of cultured tissues. From a viewpoint of engineering, therefore, the strategic construction to estimate the QC for efficacy is inevitable. BPSE, as shown in Fig.9, has focused on the tool development for spatial estimation of cells in cultured tissues(14-16).

The stereoscopic image analysis of fluorescence-labeled chondrocyte cells for cytoplasm and nucleus was performed for the quantitative determination of spatial cell distribution as well as cell aggregate size in the collagen-embedded culture. The three-dimensional histomorphometric data indicated that the cells in the gels formed aggregates by cell division, and the size of aggregates increased with elapsed culture time, revealing the mechanism of the spatial heterogeneity in cell distribution. Further development describes the morphological assessment of chondrogenic potency during a cell expanding process through serial subculturing, although the monolayer growth of chondrocytes causes dedifferentiation and results in a dysfunctional cartilage structure after transplantation, and the frequency of spherical-shaped cells in the CL gels was found to decrease along with dedifferentiation when the passaged cells were embedded in the CL gels suggesting that the morphological evaluation enables the grasping the chondrogenic potency in terms of the phenotypes and differentiate states, ultimately leading to quality control of tissue-engineered cartilage. This imaging technique, as shown in Fig.10, was applied to estimate the densities of total and proliferative cells by staining of whole nuclei and proliferative nuclei, respectively in 3-D epithelial sheets (17).

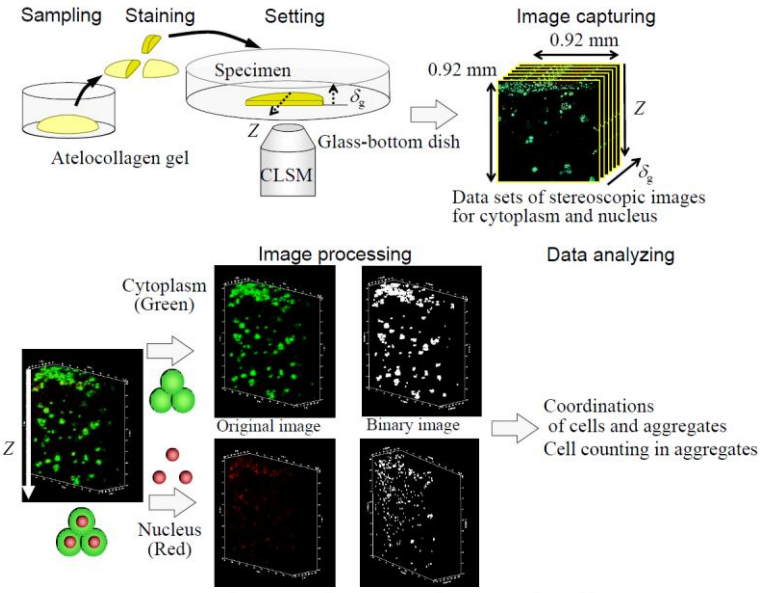

Fig.9 Experimental procedures with spatial cell distribution analyzer consisting of image capturing and its processing

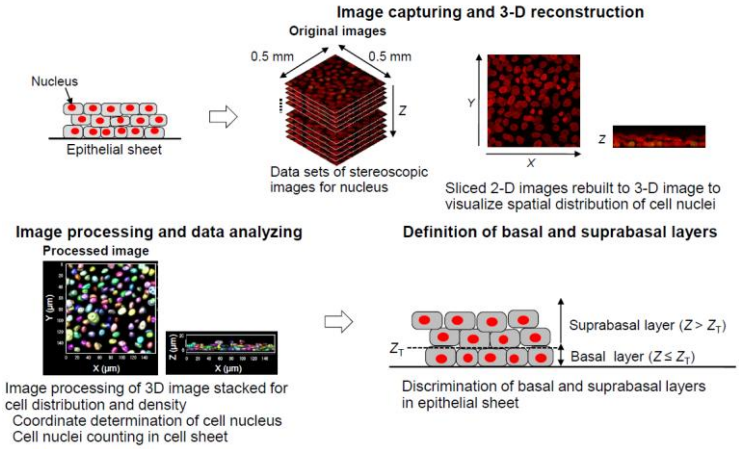

Fig.10 Experimental procedure for analyzing spatial cell nucleus distribution by means of image capturing and processing, and definition of basal layer in multilayer sheet

The cell sheet engineering is emerging as an advanced technique with preparation of scaffold-free three-dimensional (3-D) tissue, not only for transplantations, but also for *in vitro* researches. Recently autologous transplantation of multilayered myoblast sheet has been attracting attention as a new technique for curing myocardial infarction, which is associated

with the dysfunction of cardiomyocytes and irreversible cell loss. This method can overcome the disadvantages such as less take ratio of transplanted cells through the direct injection of myoblast suspension. Skeletal myoblasts, which are easy to be harvested from patients, have ability to become active, self-renew and differentiate, permitting muscle regeneration upon muscle injury. As shown in Fig.11, the sheet of myoblasts also has ability to source the cytokines which improve heart function due to paracrine system including the facilitation of angiogenesis and the attraction of progenitors on affected part. From manufacturing point of view, the system development for process and quality controls is important to be concerned, leading to the active commercialization using the cell sheets. Many researches have been tackled concerning cell source exploring, cell culture, sheet assembling, and *in vivo* animal tests. However, the method for quality control of myoblast sheet, especially for transplant efficacy, has not been systematized. The image analysis technique described above was applied to the culture system that uses multilayered sheet containing stained target cells in the basal layer and confocal laser scanning microscopy, and realizes clear observation of target cell behaviors in the vertical direction, enabling mono-dimensional analysis of vertical cell distribution inside the sheet (18). The reduced spatial dimension makes easy to analyze cell migration, compared to the full 3-D analysis required of spherically shaped aggregates. Thus, the system developed in the present study can be a powerful tool for elucidating dynamic phenomena in 3-D constructs.

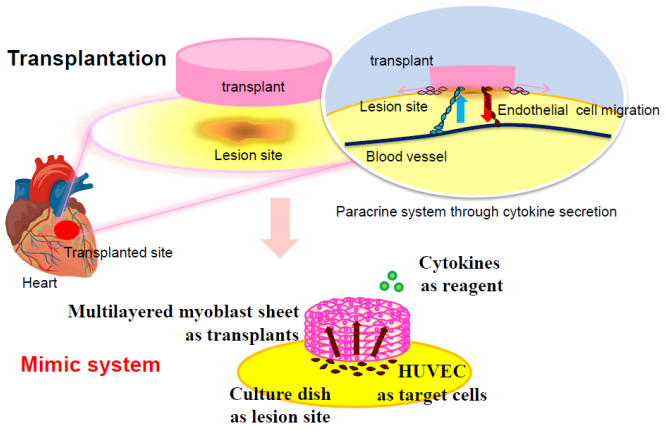

Fig.11 Conceptual drawing of new approach for quality evaluations of multilayer sheet through angiogenesis

Five-layered myoblast sheet was fabricated as a 3-D model to evaluate vertical cell migration by confocal laser scanning microscopy with image processing. And BPSE establishes the mimic system of transplantation which consists of endothelial cells (HUVECs) on culture dish as target cells on lesion site and five-layered myoblast sheet as transplants, and focus on understanding angiogenesis procedure as post transplantation. The proposed mimic system, as shown in Fig.12, expresses the formation of endothelial network formation and possesses the operational variables such as incubation time, initial density of HUVEC, myoblast density in sheet, contamination population of skeletal fibroblasts and so on. The population of skeletal myoblasts and fibroblasts,

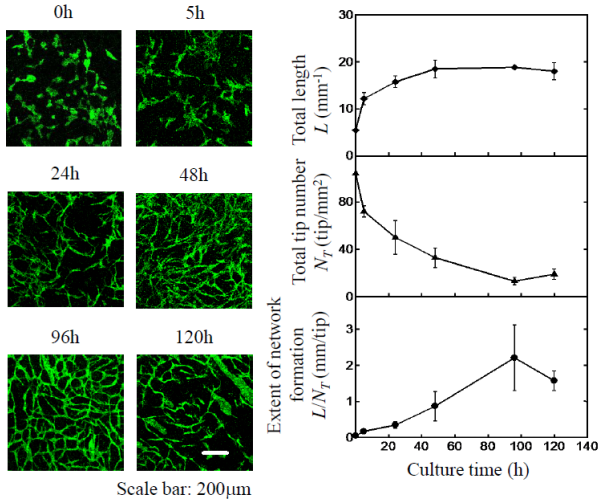

Fig.12 Profile of network formation of HUVEC in multilayered sheet

which depends on cell sources from the patients, drastically affected the HUVEC connectivity. These results suggest our mimic system can make *in vitro* quantitative estimation of angiogenesis potential for the transplants from the patients.

4. Culture Simulation group

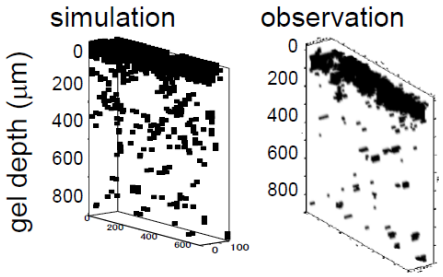

Fig.13 Spatial cell distribution in cultured cartilage at 21 days

In manufacturing, the "raw materials" of cells isolated from patients (or donors) are of heterogeneity in cell states, and the "products" of cultured tissues have variation in the required volume for individual cultured tissues (products). During the three-dimensional culture, the spatial distribution and aggregation of target cells in the cultured constructs is considered to occur owing to the limitation of oxygen and protein supplies. In addition, from a practical standpoint of cultured tissue production, the extent of cell division that depends on the states of origins such as donor age and location of harvesting biopsy becomes an obstacle to acquisition of sufficient number of cells in the 3-D cultures. The heterogeneity found in the culture process leads to the batch process with less reproducibility. These imply the production in a so-called "tailor-made process" where culture operations must be handled case by case. Therefore, the reliable and robust process is required for manufacturing the products with high quality while considering the instability and fluctuation in conditions of the raw materials.

The spatial distribution of target cells in the construct is very important information for evaluation of cultured tissues and it is desired to estimate the potentials of population doublings in the 3-D cultures. In particular, a feasible method to evaluate culture states is needed to support the operators who have to judge the cessation of 3-D cultures with useful information. To analyze the growth manner, a kinetic model is a promising tool for the prediction of spatial heterogeneity which affects the quality of cultured tissues (19).

BPSE proposed the stochastic modeling based on cellular automaton which can be introduced to express the complex dynamics of cell proliferation in the construct (19-20). As shown in Fig.13, this model revealed that the special distribution of cell aggregates depends on the culture time and cell seeding density. The profiles of cell number and aggregate distribution are attributed to the limitation of oxygen supply as well as spatial contact inhibition due to the loss of space capable for cell division, similarly to those derived from the observation data, being a useful tool to estimate the overall cell propagation and spatial cell distribution in the collagen-gel embedded culture of chondrocytes.

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