

# **Design of Biodegradable Hydrogel Microspheres to Enhance Cell Functions for Cell Aggregates**

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|-----------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>General Introduction.....</b>                                                                                                        | <b>11</b> |
| References .....                                                                                                                        | 17        |
| <br><b>PART I. Design of Gelatin Hydrogel Microspheres to Enhance Cell Viability and Functions for Mesenchymal Cell Aggregates.....</b> | <b>25</b> |
| <br><b>Chapter 1. Preparation of cell aggregates incorporating gelatin hydrogel microspheres with different sizes.....</b>              | <b>27</b> |
| 1.1 Introduction .....                                                                                                                  | 27        |
| 1.2 Methods .....                                                                                                                       | 30        |
| 1.2.1 Preparation of gelatin hydrogel microspheres with different sizes.....                                                            | 30        |
| 1.2.2 Evaluation of in vitro degradation of gelatin hydrogel microspheres with different sizes.....                                     | 31        |
| 1.2.3 Preparation of cell aggregates incorporating gelatin hydrogel microspheres with different sizes.....                              | 31        |
| 1.2.4 Measurement of live cells number in cell aggregates incorporating gelatin hydrogel microspheres with different sizes.....         | 33        |
| 1.2.5 Evaluation of cell viability in cell aggregates incorporating gelatin hydrogel microspheres with different sizes.....             | 33        |
| 1.2.6 Evaluation of cell distribution in cell aggregates incorporating gelatin hydrogel microspheres with different sizes.....          | 34        |
| 1.2.7 Measurement of cell density in cell aggregates incorporating gelatin hydrogel microspheres with different sizes.....              | 34        |
| 1.2.8 Measurement of L-lactic acid/glucose ratio of cell aggregates incorporating gelatin                                               |           |

|                                                                                                |    |
|------------------------------------------------------------------------------------------------|----|
| hydrogel microspheres with different sizes.....                                                | 35 |
| 1.2.9 Evaluation of osteogenic differentiation of cell aggregates incorporating gelatin        |    |
| hydrogel microspheres with different sizes.....                                                | 36 |
| 1.2.10 Statistical analysis.....                                                               | 36 |
| 1.3 Results.....                                                                               | 37 |
| 1.3.1 Characterization of gelatin hydrogel microspheres with different sizes.....              | 37 |
| 1.3.2 Formation of cell aggregates incorporating gelatin hydrogel microspheres with            |    |
| different sizes.....                                                                           | 38 |
| 1.3.3 Live cells number of cell aggregates incorporating gelatin hydrogel microspheres with    |    |
| different sizes.....                                                                           | 42 |
| 1.3.4 Viability of cells in cell aggregates incorporating gelatin hydrogel microspheres with   |    |
| different sizes.....                                                                           | 43 |
| 1.3.5 F-actin and cell distribution of cells in cell aggregates incorporating gelatin hydrogel |    |
| microspheres with different sizes.....                                                         | 44 |
| 1.3.6 Density of cells in cell aggregates incorporating gelatin hydrogel microspheres with     |    |
| different sizes.....                                                                           | 45 |
| 1.3.7 L-lactic acid/glucose ratio of cell aggregates incorporating gelatin hydrogel            |    |
| microspheres with different sizes.....                                                         | 47 |
| 1.3.8 Alkaline Phosphatase activity and calcium content of cells in cell aggregates            |    |
| incorporating gelatin hydrogel microspheres with different sizes.....                          | 49 |
| 1.4 Discussion .....                                                                           | 51 |
| References .....                                                                               | 56 |

## **Chapter 2. Preparation and functional evaluation of cell aggregates incorporating gelatin**

|                                                                                                                                                                       |           |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>microspheres with different degradabilities .....</b>                                                                                                              | <b>64</b> |
| 2.1 Introduction .....                                                                                                                                                | 64        |
| 2.2 Methods .....                                                                                                                                                     | 67        |
| 2.2.1 Preparation of gelatin hydrogel microspheres with different degradabilities.....                                                                                | 67        |
| 2.2.2 Evaluation of in vitro degradation of gelatin hydrogel microspheres with different degradabilities.....                                                         | 67        |
| 2.2.3 Cell culture experiments.....                                                                                                                                   | 68        |
| 2.2.4 Preparation of cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities.....                                                  | 68        |
| 2.2.5 Evaluation of cell distribution in cell aggregate incorporating gelatin hydrogel microspheres with different degradabilities.....                               | 69        |
| 2.2.6 Evaluation of degradability of gelatin hydrogel microspheres in cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities..... | 69        |
| 2.2.7 Measurement of cell density in cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities.....                                  | 70        |
| 2.2.8 Evaluation of cell viability in cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities.....                                 | 70        |
| 2.2.9 Measurement of L-lactic acid/glucose ratio of cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities.....                   | 71        |
| 2.2.10 Evaluation of Osteogenic differentiation of cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities.....                    | 72        |
| 2.2.11 Statistical analysis.....                                                                                                                                      | 72        |
| 2.3 Results.....                                                                                                                                                      | 74        |

|                                                                                                                                                                |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 2.3.1 Characterization of gelatin hydrogel microspheres .....                                                                                                  | 74 |
| 2.3.2 Formation of cell aggregates incorporating gelatin hydrogel microspheres with<br>different degradabilities .....                                         | 75 |
| 2.3.3 Distribution of cells in cell aggregates incorporating gelatin hydrogel microspheres<br>with different degradabilities .....                             | 76 |
| 2.3.4 Degradability of gelatin hydrogel microspheres in cell aggregates incorporating<br>gelatin hydrogel microspheres with different degradabilities .....    | 77 |
| 2.3.5 Cell conditions in cell aggregates incorporating gelatin hydrogel microspheres with<br>different degradabilities .....                                   | 78 |
| 2.3.6 L-lactic acid/glucose ratio of cell aggregates incorporating gelatin hydrogel<br>microspheres with different degradabilities .....                       | 80 |
| 2.3.7 Alkaline Phosphatase activity and calcium content of cell aggregates incorporating<br>gelatin hydrogel microspheres with different degradabilities ..... | 82 |
| 2.4 Discussion .....                                                                                                                                           | 86 |
| References .....                                                                                                                                               | 90 |

### **Chapter 3. Preparation of cell aggregates incorporating gelatin hydrogel microspheres**

|                                                                                                                   |           |
|-------------------------------------------------------------------------------------------------------------------|-----------|
| <b>containing bone morphogenic protein-2 with different degradabilities .....</b>                                 | <b>97</b> |
| 3.1 Introduction .....                                                                                            | 97        |
| 3.2 Methods .....                                                                                                 | 99        |
| 3.2.1 Preparation of gelatin hydrogel microspheres with different degradabilities .....                           | 99        |
| 3.2.2 Evaluation of in vitro degradation of gelatin hydrogel microspheres with different<br>degradabilities ..... | 99        |
| 3.2.3 Preparation of gelatin hydrogel microspheres containing bone morphogenic protein-2                          |           |

|                                                                                             |     |
|---------------------------------------------------------------------------------------------|-----|
| with different degradabilities.....                                                         | 100 |
| 3.2.4 Cell culture experiments.....                                                         | 100 |
| 3.2.5 Preparation of cell aggregates incorporating gelatin hydrogel microspheres            |     |
| containing bone morphogenic protein-2 with different degradabilities.....                   | 101 |
| 3.2.6 Osteogenic differentiation of MC3T3-E1 cells in cell aggregates cultured with gelatin |     |
| hydrogel microspheres containing bone morphogenic protein-2 with different                  |     |
| degradabilities.....                                                                        | 101 |
| 3.2.7 Evaluation of bone morphogenic protein-2 distribution in cell aggregates              |     |
| incorporating gelatin hydrogel microspheres containing bone morphogenic                     |     |
| protein-2.....                                                                              | 103 |
| 3.2.8 Measurement of live cells number in cell aggregates incorporating gelatin hydrogel    |     |
| microspheres containing bone morphogenic protein-2 with different                           |     |
| degradabilities.....                                                                        | 103 |
| 3.2.9 Measurement of L-lactic acid/glucose ratio of cell aggregates incorporating gelatin   |     |
| hydrogel microspheres containing bone morphogenic protein-2 with different                  |     |
| degradabilities.....                                                                        | 104 |
| 3.2.10 Evaluation of osteogenic differentiation of cell aggregates incorporating gelatin    |     |
| hydrogel microspheres containing bone morphogenic protein-2 with different                  |     |
| degradabilities.....                                                                        | 104 |
| 3.2.11 Statistical analysis.....                                                            | 106 |
| 3.3 Results.....                                                                            | 107 |
| 3.3.1 Characterization of gelatin hydrogel microspheres with different degradabilities..... | 107 |
| 3.3.2 Formation of Cell aggregates incorporating gelatin hydrogel microspheres containing   |     |
| bone morphogenic protein-2 with different degradabilities.....                              | 108 |

|                                                                                                                                                                                                                                               |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 3.3.3 BMP2 distribution in cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2.....                                                                                                             | 109 |
| 3.3.4 Live cell number and L-lactic acid/glucose ratio of cells in cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities.....                                      | 110 |
| 3.3.5 Alkaline Phosphatase activity and Runt-related transcription factor 2 mRNA expression of cells in cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities..... | 113 |
| 3.4 Discussion .....                                                                                                                                                                                                                          | 118 |
| References .....                                                                                                                                                                                                                              | 123 |

## **PART II. Design of Gelatin hydrogel or Matrigel Microspheres to Enhance Cell Viability and Functions of Epithelial Cell Aggregates.....132**

|                                                                                                                                                                       |            |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>Chapter 4. Preparation of epithelial cell aggregates incorporating matrigel microspheres to enhance proliferation and differentiation of epithelial cells.....</b> | <b>134</b> |
| 4.1 Introduction .....                                                                                                                                                | 134        |
| 4.2 Methods .....                                                                                                                                                     | 136        |
| 4.2.1 Preparation of matrigel microspheres.....                                                                                                                       | 136        |
| 4.2.2 Preparation of gelatin hydrogel microspheres.....                                                                                                               | 136        |
| 4.2.3 Preparation of matrigel-coated gelatin hydrogel microspheres.....                                                                                               | 137        |
| 4.2.4 Cell culture experiments.....                                                                                                                                   | 137        |
| 4.2.5 Preparation of cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres.....                         | 138        |

|                                                                                                                                                                                                   |     |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 4.2.6 Evaluation of cells viability in cell aggregates incorporating matrigel microspheres,<br>gelatin microspheres, and matrigel-coated gelatin microspheres.....                                | 139 |
| 4.2.7 Measurement of live cells number in cell aggregates incorporating matrigel<br>microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres.....                             | 139 |
| 4.2.8 Measurement of L-lactic acid/glucose ratio of cell aggregates incorporating matrigel<br>microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres.....                   | 140 |
| 4.2.9 Immunostaining of cell aggregates incorporating matrigel microspheres, gelatin<br>microspheres, and matrigel-coated gelatin microspheres.....                                               | 140 |
| 4.2.10 Measurement of $\beta$ -casein and E-cadherin expression in cell aggregates incorporating<br>matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin<br>microspheres..... | 141 |
| 4.2.11 Statistical analysis.....                                                                                                                                                                  | 142 |
| 4.3 Results.....                                                                                                                                                                                  | 143 |
| 4.3.1 Charactrization of matrigel microspheres, gelatin microspheres, and matrigel-coated<br>gelatin microspheres.....                                                                            | 143 |
| 4.3.2 Formation of cell aggregates incorporating matrigel microspheres, gelatin<br>microspheres, and matrigel-coated gelatin microspheres.....                                                    | 144 |
| 4.3.3 Cell viability of cell aggregates incorporating matrigel microspheres, gelatin<br>microspheres, and matrigel-coated gelatin microspheres.....                                               | 145 |
| 4.3.4 Live cells number of cell aggregates incorporating matrigel microspheres, gelatin<br>microspheres, and matrigel-coated gelatin microspheres.....                                            | 147 |
| 4.3.5 L-lactic acid/glucose ratio of cell aggregates incorporating matrigel microspheres,<br>gelatin microspheres, and matrigel-coated gelatin microspheres.....                                  | 148 |
| 4.3.6 Laminin and $\beta$ -casein expression of cell aggregates incorporating matrigel                                                                                                            |     |

|                                                                                                                                                                                |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres.....                                                                                              | 149 |
| 4.3.7 $\beta$ -casein and E-cadherin expression of cell aggregates incorporating matrigel<br>microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres..... | 151 |
| 4.4 Discussion .....                                                                                                                                                           | 155 |
| References .....                                                                                                                                                               | 159 |

## **Chapter 5. Preparation of EpH4 and 3T3L1 cells aggregates incorporating gelatin hydrogel**

|                                                                                                                                                                       |            |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>microspheres for a cell condition improvement.....</b>                                                                                                             | <b>165</b> |
| 5.1 Introduction .....                                                                                                                                                | 165        |
| 5.2 Methods .....                                                                                                                                                     | 168        |
| 5.2.1 Preparation of gelatin hydrogel microspheres.....                                                                                                               | 168        |
| 5.2.2 Cell culture experiments.....                                                                                                                                   | 169        |
| 5.2.3 Attachment of EpH4 cells to fibronectin-treated gelatin microspheres.....                                                                                       | 169        |
| 5.2.4 Preparation of EpH4/3T3L1 cells aggregates incorporating fibronectin-treated gelatin<br>hydrogel microspheres.....                                              | 170        |
| 5.2.5 Measurement of cell number of live EpH4 and/or 3T3L1 cells aggregates<br>incorporating fibronectin-treated gelatin hydrogel microspheres.....                   | 171        |
| 5.2.6 Measurement of L-lactic acid/glucose ratio of EpH4 and/or 3T3L1 cells aggregates<br>incorporating fibronectin-treated gelatin hydrogel microspheres.....        | 172        |
| 5.2.7 Evaluation of EpH4 and 3T3L1 cells distribution in EpH4/3T3L1 cells aggregates<br>incorporating fibronectin-treated gelatin hydrogel microspheres.....          | 173        |
| 5.2.8 Immunostaining of $\beta$ -casein and laminin expression of EpH4/3T3L1 cells aggregates<br>incorporating fibronectin-treated gelatin hydrogel microspheres..... | 173        |
| 5.2.9 Measurement of $\beta$ -casein expression of EpH4/3T3L1 cells aggregates incorporating                                                                          |            |

|                                                                                                                                                        |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| fibronectin-treated gelatin hydrogel microspheres.....                                                                                                 | 174 |
| 5.2.10 Statistical analysis.....                                                                                                                       | 175 |
| 5.3 Results.....                                                                                                                                       | 176 |
| 5.3.1 Charactrization of gelatin hydrogel microspheres.....                                                                                            | 176 |
| 5.3.2 Number of EpH4 cells attached onto gelatin hydrogel microspheres without or with<br>treatment of fibronectin.....                                | 176 |
| 5.3.3 Formation of EpH4 and/or 3T3L1 cell aggregates incorporating fibronectin-treated<br>gelatin hydrogel microspheres.....                           | 177 |
| 5.3.4 Number of live cells in EpH4 and/or 3T3L1 cells aggregates incorporating<br>fibronectin-treated gelatin hydrogel microspheres.....               | 180 |
| 5.3.5 L-lactic acid/ glucose ratio of EpH4 and/or 3T3L1 cells aggregates incorporating<br>fibronectin-treated gelatin hydrogel microspheres.....       | 181 |
| 5.3.6 Distribution of EpH4 and 3T3L1 cells in EpH4/3T3L1 cells aggregates incorporating<br>fibronectin-treated gelatin hydrogel microspheres.....      | 182 |
| 5.3.7 Expression of $\beta$ -casein and laminin of EpH4/3T3L1 cells aggregates incorporating<br>fibronectin-treated gelatin hydrogel microspheres..... | 184 |
| 5.4 Discussion .....                                                                                                                                   | 189 |
| References .....                                                                                                                                       | 193 |
| Summary .....                                                                                                                                          | 199 |
| List of Publications .....                                                                                                                             | 203 |
| Other Publications .....                                                                                                                               | 204 |
| Acknowledgements .....                                                                                                                                 | 205 |



## **General Introduction**

Further development of basic cell researches is highly required to clarify the molecular mechanisms of cells proliferation and differentiation. The conventional cell culture has been performed in the plastic dish of two-dimensional (2D), which is quite different from the local environment of cells in living tissues. All living tissues are not composed of plastic materials, but integrated in the three dimensional (3D) structures of multiple types of cells and extracellular matrices (ECM). Based on this background, 3D cell culture technologies have been investigated [1–5]. In the 3D cell culture, there have been two approaches of top-down and bottom-up methods to fabricate 3D tissue constructs [6]. For example, in the top-down method, 3D tissue constructs have been prepared culturing cells in biodegradable hydrogels [7], scaffolds [8], and microfabricated devices [9-11]. On the other hand, in the bottom-up method, 3D tissue constructs have been formed by a cellular layer-by-layer method of cell sheets [12, 13]. The research objective of 3D cell culture systems is to investigate the mechanism of organs development [14, 15], wound healing and fibrosis [16], cancer progression and metastasis [17], the screening of drugs, and the fabrication of tissues and organs for transplantation therapy [18].

Recently, cell aggregates have been extensively investigated in the fabrication of 3D tissue constructs. Generally, the minimum unit of body is cell, but the functional unit is an aggregate of cells. In the body, most cells exist as an aggregate in which the physiological cell–cell interaction is formed and consequently enhances their survival and functions [19]. For example, embryonic stem cells generally aggregate to form an embryoid body, and then initiate their differentiation into different cell lineages [20].

The aggregation of liver cells to form a spheroid is necessary to enhance the metabolic activity [21]. Looking at the structure of body tissues, such as liver and bone, the cell aggregates function as the minimum unit [22]. The organ fundamentally develops to form from epithelium and mesenchyme by the epithelial-mesenchymal interactions during the early stage of morphogenesis [23–27]. Taken together, the fabrication of cell aggregates is one of the strategies suitable to mimic in vivo 3D tissues conditions.

However, there are some technological problems to be improved for the culture of cell aggregates. One of them is the death of cells in the center of aggregates with a large size, because of the lack of oxygen and nutrients supplies [28]. The cell death often makes it practically difficult to continue the in vitro cell culture experiments over a long time period, although the long-term culture is required for basic researches of cell differentiation and organization.

Therefore, it is necessary to create technology or methodology for 3D cell cultures of poor oxygen and nutrients supplies to cells in aggregates. There were some methodologies to improve the oxygen and nutrients supplies to cells. For example, co-culture of endothelial cells with target tissue cells in 3D culture [29, 30] and embodiment of angiogenesis growth factors in 3D scaffolds for prolonged release [31, 32], because oxygen and nutrients are generally delivered through the vascular networks. For example, a 3D cell culture is conducted with a polydimethylsiloxane (PDMS) membrane of gas permeability [33].

In this thesis, as one trial to tackle the problem, the incorporation of hydrogel materials in the aggregate protected the aggregate from the lack of oxygen and nutrients, because oxygen and nutrients can be normally permeated through the water phase of hydrogel matrix [34]. As the hydrogel material, gelatin hydrogel microspheres were

selected. A previous study showed that the incorporation of gelatin hydrogel microspheres protected the aggregate of rat bone marrow-derived mesenchymal stem cells (MSC) from the lack of nutrients and oxygen, resulting in enhanced MSC proliferation in aggregates [35].

Gelatin is a biodegradable biomaterial which has been extensively used for medical, pharmaceutical, and cosmetic applications. Its biosafety has been proven through the long-term practical usage [36]. Gelatin hydrogels of different shapes can be formulated, while their feasibility as cell culture substrates [37–39] and cell scaffolds for tissue regeneration [40–44] or as carriers of growth factors and drugs [45–52] has been experimentally demonstrated. Gelatin hydrogel can release growth factors to induce tissue regeneration [50, 53–55]. Based on the availability and the easiness of hydrogel formulation, gelatin was selected as the hydrogel material.

Several researches have been reported on the cell aggregates incorporating the microspheres of polymers, such as gelatin, poly (lactic-co-glycolic acid) (PLGA), and alginate [56–58]. It has been demonstrated that the PLGA and alginate microspheres do not always have suitable property for cell adhesion. On the contrary, gelatin hydrogel microspheres (GM) have a good cell adhesion property. In this thesis, based on the findings, gelatin hydrogel microspheres were prepared to evaluate the cell functions in cell aggregates. In addition, the gelatin hydrogel microspheres have an ability to allow the controlled release of growth factors as reported previously [50, 54, 55]. In addition, there have been a few reports to investigate the effect of microspheres properties on the cells functions. The objective of this thesis is to design and prepare biodegradable hydrogel microspheres aiming at the enhancement of the survival and functions of cell aggregates.

In PART I, mesenchymal cell aggregates incorporating gelatin hydrogel microspheres (GM) with different sizes, degradabilities, or containing bone morphogenic protein-2 (BMP2) were designed. The viability, proliferation and differentiation of mesenchymal of MC3T3-E1 cells in cell aggregates incorporating GM with different sizes, degradabilities, or containing BMP2 were evaluated.

Chapter 1 is concerned with the effect of GM size on the cells viability and functions in the MC3T3-E1 cell aggregates incorporating GM. When compared at the same surface area of GM used, the ratio of L-lactic acid production/glucose consumption and the cell density of cell aggregates were significantly lower for the GM of larger sizes than those of smaller sizes. Alkaline phosphatase (ALP) activity and calcium content assays to evaluate the osteogenic differentiation of MC3T3-E1 cells demonstrated that both the values were significantly higher. In the long-term culture, the incorporation of larger size GM gave cells a better condition for oxygen and nutrients supply into the cell aggregates, resulting in an enhanced osteogenic differentiation of cells.

Chapter 2 is concerned with the effect of GM degradability on the cells viability and functions in MC3T3-E1 cell aggregates incorporating GM. The longer remaining time period of slow-degraded GM in the cell aggregates at large amounts of resulted in the higher proliferation of MC3T3-E1 cells. The ratio of L-lactic acid /glucose ratio of MC3T3-E1 cells was significantly lower than that of aggregates incorporating fast-degraded GM. The ALP activity and calcium content of MC3T3-E1 cells were higher for MC3T3-E1 cells aggregates incorporating slow-degraded GM. The incorporation of slow-degraded GM gave cells in the aggregate better culture conditions, resulting in improved survival, proliferation, and osteogenic differentiation.

Chapter 3 is concerned with the survival and functions of cells in MC3T3-E1 cell aggregates incorporating GM with different degradabilities which contain bone morphogenic protein-2 (BMP2). The runt-related transcription factor 2 (RUNX2) messenger ribonucleic acid (mRNA) expressions, ALP activity, and calcium content of MC3T3-E1 cells in cell aggregates were evaluated for their osteogenic differentiation. GM containing BMP2 (GM-BMP2) incorporated in the cell aggregates enhanced the osteogenic differentiation of cells compared with free BMP2 added externally. The degradability of GM-BMP2 affected the extent of osteogenic differentiation. In PART II, epithelial cell aggregates incorporating matrigel microspheres (MM) and/or GM were prepared. In addition, epithelial/mesenchymal cells aggregates incorporating GM were prepared. The proliferation and differentiation of epithelial EpH4 cells in cell aggregates incorporating MM and/or GM were evaluated.

Chapter 4 is concerned with the effect of matrigel microspheres (MM), GM, and matrigel-coated GM on the proliferated and biological functions of epithelial cells in EpH4 cell aggregates incorporating the microspheres. Higher EpH4 cells proliferation was observed for cell aggregates incorporating MM, GM, and matrigel-coated GM compared with the conventional 3D culture method. When examined to evaluate the epithelial differentiation of EpH4 cells, the  $\beta$ -casein expression was significantly higher for the cell aggregates incorporating MM than that of aggregates incorporating GM and matrigel-coated GM or the conventional 3D culture method. The proliferation and differentiation of mammary epithelial EpH4 cells were promoted by the incorporation of MM.

Chapter 5 is undertaken to prepare the 3D aggregates of mouse mammary epithelial EpH4 and mouse preadipocyte 3T3L1 cells in the presence of GM and evaluate the

effect of GM presence on the survival and functions of cells in the 3D cell aggregates. GM were treated with fibronectin (FN) for improvement of EpH4 cells attachment to GM. When 3T3L1 cells were incubated with the original and GM-preincubated EpH4 cells in the presence of the FN-treated GM, the number of live cells in the aggregates was significantly high compared with that for the absence of FN-treated GM. In addition, higher  $\beta$ -casein expression level of EpH4 cells in EpH4/3T3L1 cells aggregates in the presence of FN-treated GM was observed than that of cells in the absence of FN-treated GM. Laminin secretion was also promoted for the cells aggregates cultured with FN-treated GM. The presence of FN-treated GM in the EpH4/3T3L1 cells aggregates gave a better condition to cells, resulting in an enhanced generation of  $\beta$ -casein from EpH4 cells in the aggregates.

In summary, this thesis describes the importance and necessity of hydrogel microspheres to enhance cells survival and functions in cell aggregates. It is concluded that the properties of hydrogel microspheres were important to enhance the survival and functions of cell aggregates.

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## **PART I**

### **Design of Gelatin Hydrogel Microspheres to Enhance Cell Viability and Functions for Mesenchymal Cell Aggregates**



## **Chapter 1**

### **Preparation of cell aggregates incorporating gelatin hydrogel microspheres with different sizes**

#### **1.1 Introduction**

Recently, the three-dimensional (3D) cultures of cell aggregates have been increasingly noted to enhance their cell functions and investigate the molecular mechanisms of cell proliferation and differentiation [1–4]. The conventional cell culture has been performed in two dimensions, which is different from the local environment of cells in living tissues. In the body tissue, most of cells exist in the 3D structure where the cell–cell interaction is physiologically promoted to enhance the cell survival and functions [5]. Embryonic stem cells generally aggregate to form an embryoid body and consequently initiate the cell differentiation into different lineages [6]. The aggregation of liver cells to form a spheroid is necessary to enhance the metabolic activity [7]. Cell aggregates produce extracellular matrix proteins more efficiently than single cells [8]. It is reported that it is the minimum unit of body structure, but that of biological function is the aggregates of cells [9]. The examples strongly suggest that a 3D aggregation allows cells to promote the cell–cell interactions, resulting in enhanced biological formations of cells. It is, therefore, necessary to develop 3D cell culture technologies. However, there are some technological problems to be resolved in the culture of cell aggregates. One of them is that as the size of cell aggregates became larger, cells in the center of large aggregates readily often become weaker or die due to weaker and the lack of oxygen and nutrients supplies [10, 11]. This problem often causes difficulty to continue the in vitro cell culture over a long time period, although the long-term culture

is required for the basic research of cell differentiation and organization. The previous study demonstrated that the incorporation of gelatin hydrogel microspheres (GM) prevents rat bone marrow-derived mesenchymal stem cells (MSC) in their aggregates from their death, but permit the enhancement of cell proliferation and osteogenic differentiation [12]. This is because the water phase of hydrogel matrix facilitates to supply oxygen and nutrients to cells [13].

Several researches have been reported on the cell aggregates incorporating the microspheres of gelatin, poly (lactic-co-glycolic acid) (PLGA), and alginate [11, 14-21]. Gelatin is a biodegradable biomaterial which have been extensively used for medical, pharmaceutical, and cosmetic applications. Its biosafety has been proven through the long-term practical usage [22]. Gelatin is used as one of the materials with a good cell and tissue compatibilities. Hydrogels of different shapes can be formulated, and the hydrogel feasibility in cell culture substrates [23–24], and cell scaffolds for tissue regeneration [25–31], or the carriers of growth factors and drugs [32–38], has been experimentally demonstrated. Gelatin hydrogels are effective in the controlled release of growth factors for tissue regeneration [39–42]. Based on the availability and the easiness of hydrogel formulation, gelatin was selected as the hydrogel material in this study.

The objective of this study is to investigate the effect of microspheres size on the cells viability and functions in the aggregates incorporating GM. To this end, GM with different sizes, but the same degradability was prepared by tuning the crosslinking conditions of gelatin. After MC3T3-E1 cell were incubated with GM to form the cell aggregates, the viability, metabolic activity, and osteogenic differentiation of MC3T3-E1 cells were evaluated in terms of microspheres size. We examine the

influence of GM number, volume, and surface area on the cell function in cell aggregates.

## 1.2 Materials and methods

### 1.2.1 Preparation of gelatin hydrogel microspheres with different sizes

Gelatin hydrogel microspheres (GM) were prepared by the chemical crosslinking of gelatin in a water-in-oil emulsion state according to the method previously reported [34]. Briefly, an aqueous solution (20 ml) of 10 wt% gelatin (isoelectric point 5.0, weight-averaged molecular weight = 1,00,000, Nitta Gelatin Inc., Osaka, Japan) was preheated at 40 °C, and then added dropwise using a pipette or injection needle 27G into 600 ml of olive oil (Wako Ltd, Osaka, Japan) at 40 °C, followed by stirring at 150 or 400 rpm for 10 min to prepare the water-in-oil emulsion. The emulsion temperature was decreased to 4 °C for the natural gelation of gelatin solution to obtain non-crosslinked hydrogel microspheres. The resulting gelatin hydrogel microspheres were washed three times with cold acetone in combination with centrifugation (5000 rpm, 4 °C, 5 min) to completely exclude the residual oil. Then, GM were fractionated by size using sieves with apertures of 20, 32, 53 , 75, and 105  $\mu\text{m}$  (Iida Seisakusyo Ltd, Osaka, Japan) and air dried at 4 °C. The non-crosslinked and dried GM (200 mg) were treated in a vacuum oven at 140 °C and 0.1 Torr for the dehydrothermal crosslinking of gelatin for various time (Table 1) according to the method previously reported [34]. The pictures of GM in the water swollen state were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan). For each sample, the size of 100 microspheres was measured using the computer program Image J (NIH Inc., Bethesda, USA) to calculate the average size.

Table 1. Preparation and characterization of GM by dehydrothermal crosslinking.

| The size of GM<br>dispersed in acetone ( $\mu\text{m}$ ) | The size of GM<br>dispersed in water ( $\mu\text{m}$ ) | Crosslinking<br>time (hr) |
|----------------------------------------------------------|--------------------------------------------------------|---------------------------|
| 2.9 $\pm$ 0.3 <sup>a)</sup>                              | 4.5 $\pm$ 1.7                                          | 192                       |
| 9.0 $\pm$ 4.0                                            | 17.0 $\pm$ 6.7                                         | 96                        |
| 22.0 $\pm$ 5.0                                           | 50.0 $\pm$ 18.0                                        | 48                        |
| 39.0 $\pm$ 9.0                                           | 103.0 $\pm$ 25.0                                       | 48                        |
| 60.0 $\pm$ 12.0                                          | 141.0 $\pm$ 26.0                                       | 48                        |
| 86.0 $\pm$ 18.0                                          | 194.0 $\pm$ 33.0                                       | 48                        |

a) mean  $\pm$  SE

### ***1.2.2 Evaluation of in vitro degradation of gelatin hydrogel microspheres with different sizes***

GM with different sizes (5 mg) were fully swollen by 750  $\mu\text{l}$  of double distilled water (DDW) in 2 ml tube (Thermo Inc., Walsam, USA) for 1 hr at 37  $^{\circ}\text{C}$ . Then, 750  $\mu\text{l}$  of 2 M hydrochloric acid (HCl) (Nacalai Tesque Inc., Kyoto, Japan) was added and incubated at 37  $^{\circ}\text{C}$  until the first sampling time. Next, the tube was centrifuged at 5,000 rpm, 37  $^{\circ}\text{C}$  for 5 min and 200  $\mu\text{l}$  of supernatant was sampled. After that, 200  $\mu\text{l}$  of 1 M HCl was added and incubated at 37  $^{\circ}\text{C}$  at different time intervals. To measure the amount of gelatin degraded by HCl, the absorbance of supernatant sampled was measured at 260 nm by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech Inc., Cambridge, England).

### ***1.2.3 Preparation of cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

Poly(vinyl alcohol) (PVA, degree of polymerization = 1,800 and 88 mol%

saponification) kindly supplied from Unichika Ltd (Tokyo, Japan) was dissolved in 1X Dulbecco's phosphate-buffered saline solution (PBS, Nissui Ltd, Tokyo, Japan) to give a concentration of 1 wt%. The PVA solution (100  $\mu$ l /well) was added to each well of 96-multiwell culture plate with either flat- or round-bottomed (U-bottomed) wells and incubated at 37  $^{\circ}$ C for 15 min. Then, the solution was removed by aspiration and the wells were washed twice with PBS (100  $\mu$ l/well). MC3T3-E1 preosteoblasts were cultured alpha minimum essential medium ( $\alpha$ MEM) (Invitrogen Inc., Carlsbad, USA) supplemented with 10 vol% fetal calf serum (FCS) (Thermo Inc., Waltham, USA), penicillin (50 U/ml), and streptomycin (50 U/ml) (standard medium) and cultured at 37  $^{\circ}$ C in a 95% air-5% carbon dioxide atmosphere. The culture medium was changed every 2 days and confluent cells were subcultured through trypsinisation. GM and MC3T3-E1 cells were separately suspended in the standard medium. The GM suspension or GM-free medium (50  $\mu$ l/well) was added to the PVA-coated wells in various conditions (Table 2), followed by the addition of MC3T3-E1 suspension ( $2 \times 10^4$  cells/ml, 50  $\mu$ l/well). The experiment of cell aggregates formation was performed in different conditions: the same number, volume, surface area of GM were applied to cells. The volume of GM was calculated by the following equation; The volume =  $\frac{4}{3} \times \pi \times (\text{Size}/2)^3 \times \text{Number of GM}$ . The surface area of GM was calculated by the following equation; The surface area =  $4 \times \pi \times (\text{Size}/2)^2 \times \text{Number of GM}$ . Experiments of each sample were performed for three wells unless mentioned otherwise. The pictures of cell aggregates with or without the GM incorporation were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan).

Table 2. Experimental condition of GM used to form cell aggregates.

| Size ( $\mu\text{m}$ ) | in the same number case:<br>Number of GM used<br>(microspheres/well) | in the same volume case:<br>Number of GM used<br>(microspheres/well) | in the same surface case:<br>Number of GM used<br>(microspheres/well) |
|------------------------|----------------------------------------------------------------------|----------------------------------------------------------------------|-----------------------------------------------------------------------|
| 4.5 $\pm$ 1.7          | $1.0 \times 10^3$                                                    | $1.2 \times 10^7$                                                    | $5.0 \times 10^5$                                                     |
| 17.0 $\pm$ 6.7         | $1.0 \times 10^3$                                                    | $2.3 \times 10^5$                                                    | $4.0 \times 10^4$                                                     |
| 50.0 $\pm$ 18.0        | $1.0 \times 10^3$                                                    | $9.0 \times 10^3$                                                    | $4.0 \times 10^3$                                                     |
| 103.0 $\pm$ 25.0       | $1.0 \times 10^3$                                                    | $1.0 \times 10^3$                                                    | $1.0 \times 10^3$                                                     |
| 141.0 $\pm$ 26.0       | $1.0 \times 10^3$                                                    | $4.0 \times 10^2$                                                    | $6.0 \times 10^2$                                                     |
| 194.0 $\pm$ 34.0       | $1.0 \times 10^3$                                                    | $1.5 \times 10^2$                                                    | $3.0 \times 10^2$                                                     |

#### ***1.2.4 Measurement of live cells number in cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

The number of live cells in MC3T3-E1 cell aggregates without or with the GM incorporation was determined by counting the number of cell nuclei after the crystal violet staining [43]. Briefly, a mixed solution of 0.2 M citric acid and 0.2 wt% crystal violet was added (100  $\mu\text{l}$ /well) to each well of multiwell plate 4, 7, 14, and 21 days after MC3T3-E1 cell culture without or with GM. After the crystal violet staining, the cells were lysed in 0.1 wt% Triton X-100 in PBS at 37  $^{\circ}\text{C}$  overnight to extract the nuclei from the cells. After pipetting, the nuclei collected were viewed under a microscope (CKX31-11PHP, Olympus Ltd, Tokyo, Japan) and counted in a hemocytometer (OneCell Inc., Hiroshima, Japan). The nuclei of live cells are generally round, while dead cell nuclei are irregularly shaped. Based on the nucleus shape, live cells can be distinguished from dead cells to assess the number of live cells.

#### ***1.2.5 Evaluation of cell viability in cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

Live/dead assays were conducted using Live/Dead<sup>®</sup> Viability/Cytotoxicity assay kit (Invitrogen Inc., Carlsbad, CA) according to the manufacture's protocol. At 21 days in culture, cell aggregates without or with the GM incorporation were rinsed with PBS, and then incubated with a solution containing 2  $\mu$ M calcein AM and 4  $\mu$ M EthD-1 in PBS for 30 min at 37 °C in the dark, followed by viewing through the confocal laser scanning microscope (FV1000D, Olympus Ltd., Tokyo, Japan).

#### ***1.2.6 Evaluation of cell distribution in cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

MC3T3-E1 cells were similarly cultured with GM to form the cell aggregates. After incubation for 21 days, the aggregates were fixed with 4 % paraformaldehyde at 4 °C for 1 hr and embedded in an optimal cutting temperature compound (Sakura Finetek Japan Co. Ltd, Tokyo, Japan) and frozen using liquid nitrogen. The frozen samples were sectioned using a cryotome (CM3050S, Leica Microsystems Inc., Wetzlar, Germany) and stained with Alexa Fluor<sup>®</sup> 488 Phalloidin (Thermo Fisher Scientific Inc., Massachusetts, USA) and TO-PRO-3 (Molecular Probes Inc., Eu-gene, USA). The sections of 10  $\mu$ m thickness were viewed in a confocal laser scanning microscope (FV1000D, Olympus Ltd, Tokyo, Japan).

#### ***1.2.7 Measurement of cell density in cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

The cell density was calculated by following equation; cell density = number of cells in cell aggregate/volume of cell aggregate without or with GM incorporation (cells/cm<sup>3</sup>).

The number of cells in cell aggregate incorporating GM was determined by the fluorometric quantification of cellular DNA according to the method reported by Rao et al. [44]. Briefly, the samples were lysed in 30 mM sodium citrate-buffered saline solution (SSC) (pH 7.4) containing 0.2 mg/ml sodium dodecylsulfate (SDS) at 37 °C for 12 hr with occasional mixing. The cell lysate (30 µl) was mixed with a dye solution (70 µl; 30 mM SSC, 10 µg/mL Hoechst 33258 dye) and the fluorescent intensity of mixed solution was measured in a fluorescence spectrometer (F-2000, HITACHI Ltd, Tokyo, Japan) at the excitation and emission wavelengths of 355 and 460 nm, respectively. The calibration curve between the DNA and cell number was prepared by use of cells of known numbers.

#### ***1.2.8 Measurement of L-lactic acid/glucose ratio of cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

MC3T3-E1 cells were cultured without or with the GM incorporation similarly to form the cell aggregates. The amount of glucose consumed by MC3T3-E1 cell aggregates was determined by measuring the change of glucose concentration in the culture medium using a Glutest Neo Super test kit (Sanwa kagaku kenkyusyo Co. Ltd, Kyoto, Japan) 21 days after incubation. Next, the amount of L-lactic acid produced by MC3T3-E1 cell aggregates was determined with an E-kit (R-Biopharm AG Co. Ltd, Germany) after 21 days incubation. The number of live cells was determined by the crystal violet staining method as described above and used to normalize the amount of glucose consumption and L-lactic acid produced to the number of live cells. The L-lactic acid/glucose ratio was calculated as a measure of the metabolic activity of cells [45].

### ***1.2.9 Evaluation of osteogenic differentiation of cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

MC3T3-E1 cells were cultured without or with GM incorporation similarly to form the cell aggregates. As a control, MC3T3-E1 cells were cultured in the flat-bottomed wells of 96-multiwell culture plates at a density of  $1 \times 10^3$  cells per well. The cell aggregates were cultured for 7 and 21 days in an osteogenic differentiation medium of  $\alpha$ MEM containing 10 mM  $\beta$ -glycerophosphate, 0.2 mM L-ascorbic acid 2-phosphate (Sigma inc., Poole, UK), 10 nM dexamethasone, and 10 vol% FCS. The cell aggregates without or with GM incorporation was lysed by 30 mM SSC (pH 7.4) containing 0.2 mg/ml SDS at 37 °C for 12 hr with occasional mixing.

The alkaline phosphatase (ALP) activity of MC3T3-E1 cells was determined using a Lab Assay ALP kit (Wako Ltd, Osaka, Japan) according to the procedure previously reported [12]. The cell lysate was incubated in 1M HCl solution for 24 hr at 4°C. The calcium amount of HCl solution was determined using a calcium E-HA kit (Wako Ltd, Osaka, Japan) according to the manufacture's protocol.

### ***1.2.10 Statistical analysis***

All the statistical data are expressed as the mean  $\pm$  standard error of the mean (SEM). The data were analyzed by t-test to determine the statistical significance of differences between two mean values, which was accepted at  $<0.05$ .

## 1.3 Results

### 1.3.1 Characterization of gelatin hydrogel microspheres with different sizes

Figure 1 shows the typical microscopic pictures of GM fractioned by different sieves. The microspheres were of spherical shape and had a smooth surface. The sizes of microspheres in the swollen condition were  $4.5 \pm 1.7$ ,  $17.0 \pm 6.7$ ,  $50.0 \pm 18.0$ ,  $103.0 \pm 25.0$ ,  $141.0 \pm 26.0$  and  $194.0 \pm 34.0$   $\mu\text{m}$ .

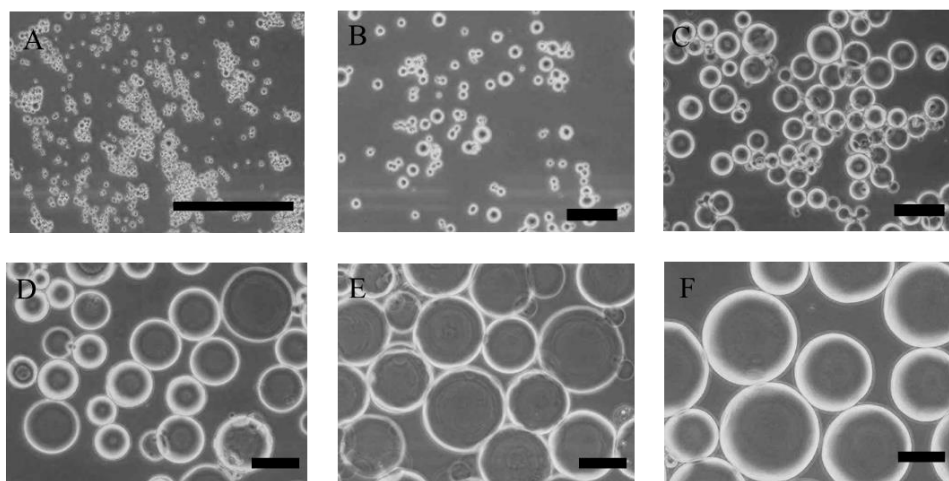


Figure 1. Light microscopic pictures of GM dispersed in water. GM were prepared by dehydrothermal crosslinking at 140 °C for 192 (A), 96 (B), 48 (C), 48 (D), 48 (E), and 48 hr (F). Scale bar. 100  $\mu\text{m}$ .

Figure 2 shows the in vitro degradation profiles of GM prepared by various conditions of dehydrothermal crosslinking. The similar degradation profile of GM was observed although their size was different.

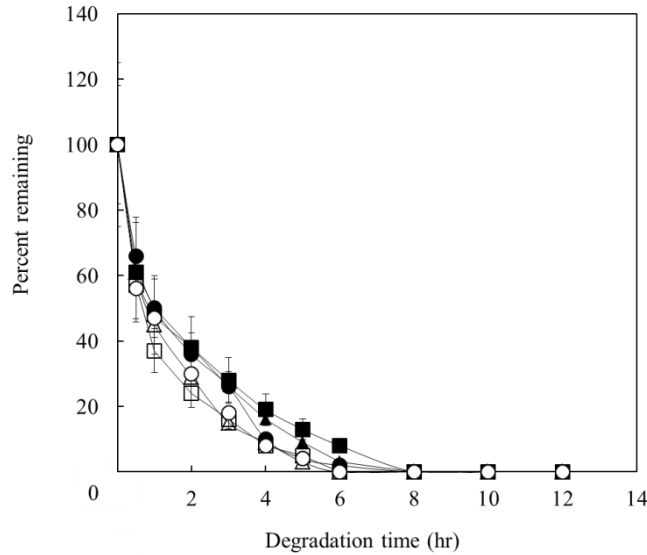


Figure 2. In vitro degradation profiles of GM with size of 4.5 (○) , 17.0 (△) , 50.0 (□), 103.0 (●), 141.0 (▲), and 194. 0 μm (■) .

### 1.3.2 Formation of cell aggregates incorporating gelatin hydrogel microspheres with different sizes

Figure 3 shows the light microscopic pictures of MC3T3-E1 cell aggregates 1, 3, 5, 7, 14 and 21 days after incubation without or with the GM incorporation at the same number of GM added. The time period to form cell aggregates incorporating GM prolonged with an increase in the GM size.

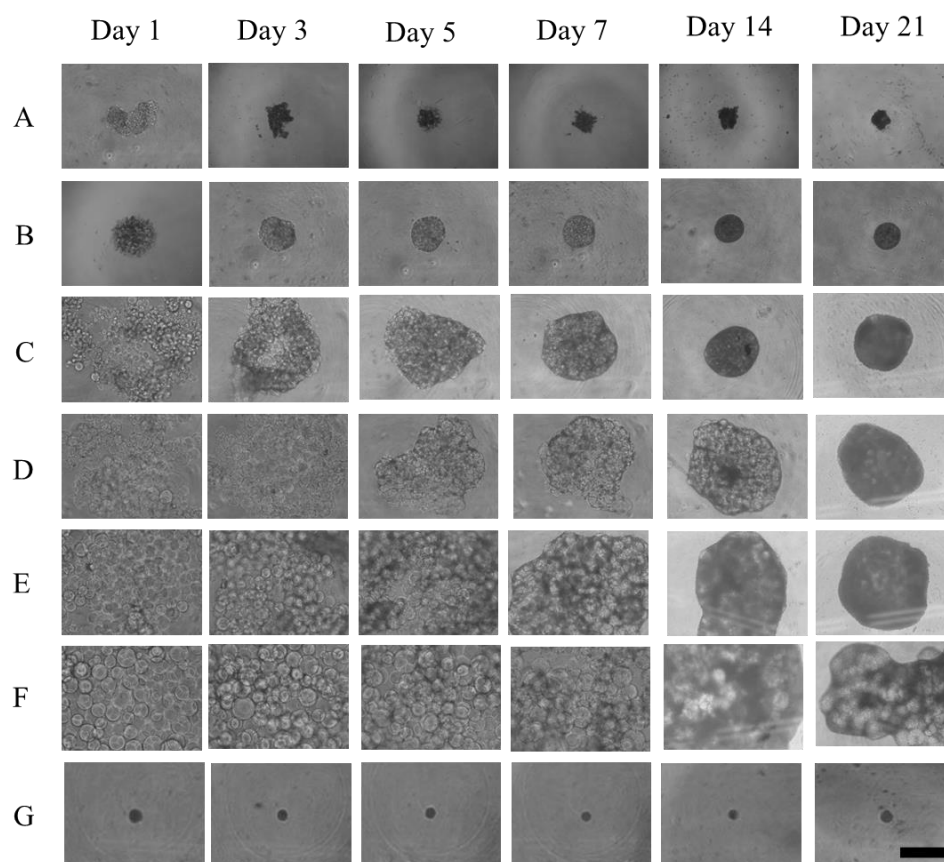


Figure 3. Light microscopic pictures of MC3T3-E1 cell aggregates 1, 3, 5, 7, 14 and 21 days after incubation of cells with (A-F) or without GM (G). The cells were cultured at the same number of GM with sizes of 4.5 (A), 17.0 (B) , 50.0 (C), 103.0 (D), 141.0 (E), and 194. 0  $\mu\text{m}$  (F). Scale bar. 100  $\mu\text{m}$ .

Figure 4 shows the microscopic pictures of MC3T3-E1 cell aggregates 1, 3, 5, 7, 14 and 21 days after incubation without or with the GM at the same volume of GM added. The time period to form cell aggregates incorporating GM shortened with an increase in the GM size.

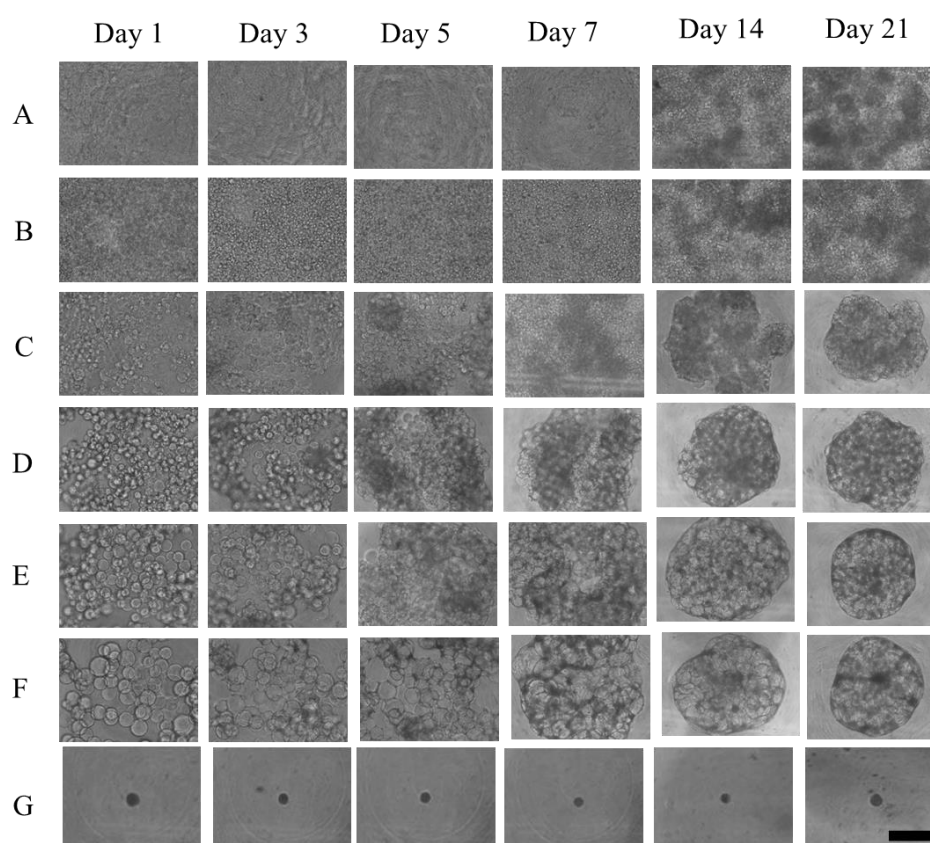


Figure 4. Light microscopic pictures of MC3T3-E1 cell aggregates 1, 3, 5, 7, 14 and 21 days after incubation of cells with (A-F) or without GM (G). The cells were cultured at the same volume of GM with sizes of 4.5 (A) , 17.0 (B) , 50.0 (C), 103.0 (D), 141.0 (E), and 194. 0  $\mu\text{m}$  (F). Scale bar. 100  $\mu\text{m}$ .

Figure 5 shows the microscopic pictures of MC3T3-E1 cell aggregates 1, 3, 5, 7, 14 and 21 days after incubation without or with the GM at the same surface area of GM added. There was no different in the time period to form cell aggregates incorporating GM among different GM.

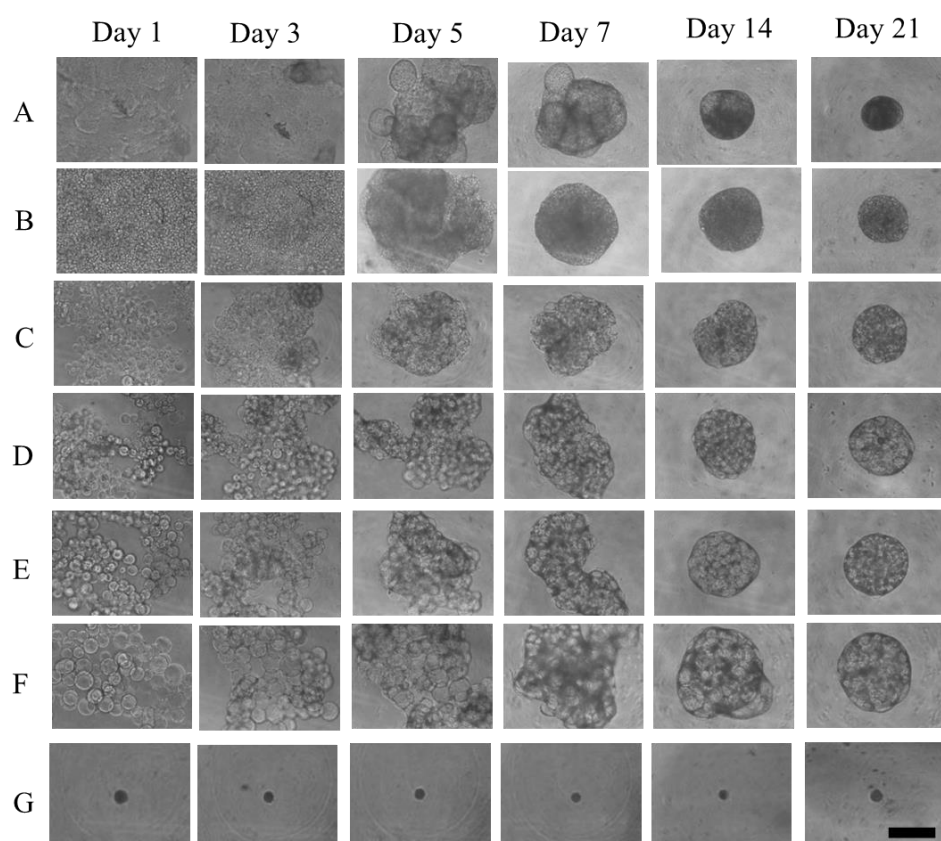


Figure 5. Light microscopic pictures of MC3T3-E1 cell aggregates 1, 3, 5, 7, 14 and 21 days after incubation of cells with (A-F) or without GM (G). The cells were cultured at the same surface area of GM with sizes of 4.5 (A), 17.0 (B), 50.0 (C), 103.0 (D), 141.0 (E), and 194.0  $\mu\text{m}$  (F). Scale bar. 100  $\mu\text{m}$ .

### ***1.3.3 Live cells number of cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

Figure 6 shows the number of live MC3T3-E1 cells 4, 7, 14 and 21 days after incubation with or without GM. The cells were cultured with GM at the same number, volume, and surface area of microspheres used. The number of live cells in GM-free cell aggregates did not increase. In contrast, the number of live cells in cell aggregates with the GM incorporation increased until to complete the aggregates. When the number of GM added was same, as the size of GM became larger, the number of cells in cell aggregates incorporating GM increased. As the same volume of GM added, oppositely, the number of cells in cell aggregates incorporating GM decreased with the GM size. Comparing at the same surface area of GM, the number of cells in cell aggregates incorporating GM tended to increase as the size of GM became larger, but the increase was not significant.

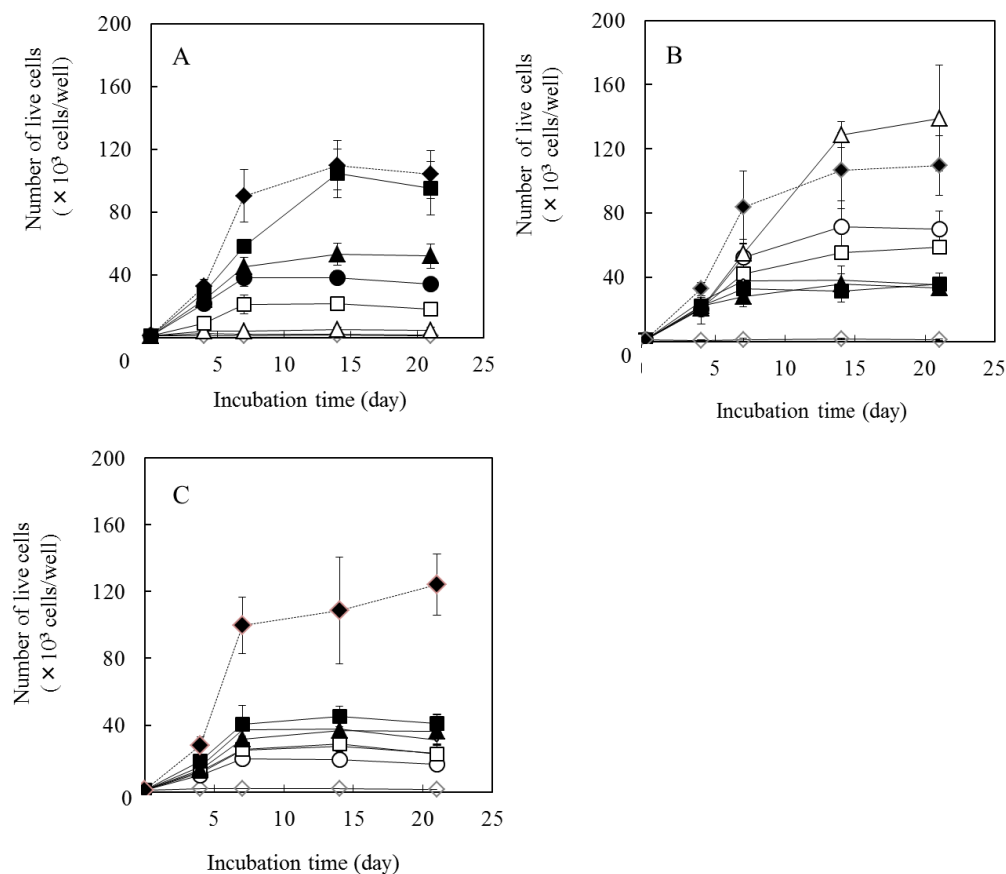


Figure 6. The number of live MC3T3-E1 cells 4, 7, 14 and 21 days after incubation with or without GM. The cells were cultured with GM at the same number (A), volume (B), and surface area of microspheres used (C). The cells were cultured with sizes of 4.5 (○), 17.0 (△), 50.0 (□), 103.0 (●), 141.0 (▲), and 194.0  $\mu\text{m}$  (■) or without GM (◇). The cells were also as the monolayer culture without GM (◆).

#### 1.3.4 Viability of cells in cell aggregates incorporating gelatin hydrogel microspheres with different sizes

Figure 7 shows the live/dead assay pictures of cell aggregates 21 days after incubation without or with GM incorporation. For GM-free cell aggregates, cells

present in the center of cell aggregates were dead. On the contrary, cells present in cell aggregates survived for cell aggregates incorporating GM. The survival extent tended to become high as the GM size was larger.

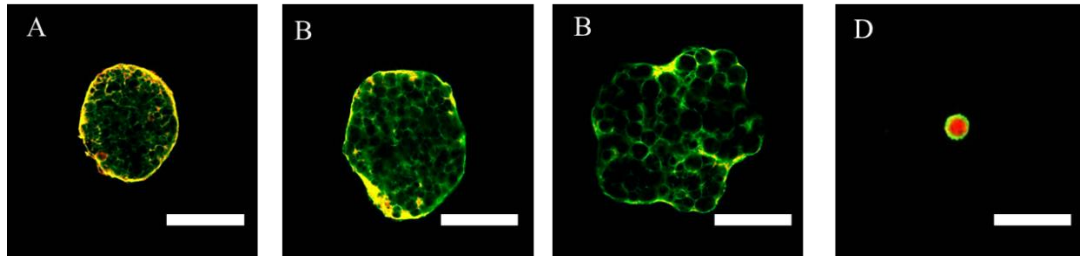


Figure 7. Live/dead assay of MC3T3-E1 cell aggregates 21 days after incubation of cells with (A-C) or without GM (D). The cells were cultured at the same surface area of GM with sizes of 17.0 (A) , 50.0 (B), and 103.0 μm (C). Scale bar. 500 μm.

### ***1.3.5 F-actin and cell distribution of cells in cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

Figure 8 shows the microscopic pictures of F-actin and nuclei of MC3T3-E1 cell aggregates 21 days after incubation with or with GM incorporation at the same surface area of GM added. As the size of GM became larger, the cell density in cell aggregates incorporating GM decreased. The shape of cell was round or ellipsed at the GM size of 17.0 μm. On the other hand, the shape of cells was elongated at the GM size of 103.0 μm.

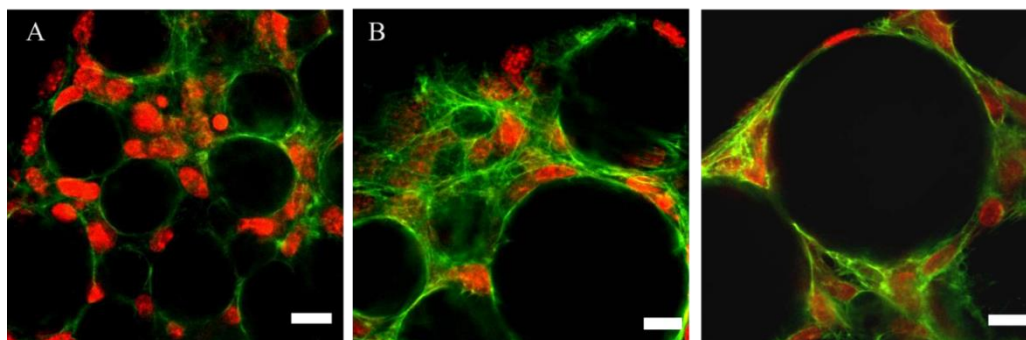


Figure 8. F-actin and nuclei of MC3T3-E1 cell aggregates 21 days after incubation of cells with or without GM. The cells were cultured at the same surface area of GM with sizes of 17.0 (A) , 50.0 (B), and 103.0  $\mu\text{m}$  (C). Scale bar. 10  $\mu\text{m}$ .

### ***1.3.6 Density of cells in cell aggregates incorporating gelatin hydrogel microspheres with different sizes***

Figure 9 shows the cell density of MC3T3-E1 cells in cell aggregates 21 days after incubation with or without GM incorporation at the same surface area of GM added. As the size of GM became larger, the cell density in cell aggregates incorporating GM decreased.

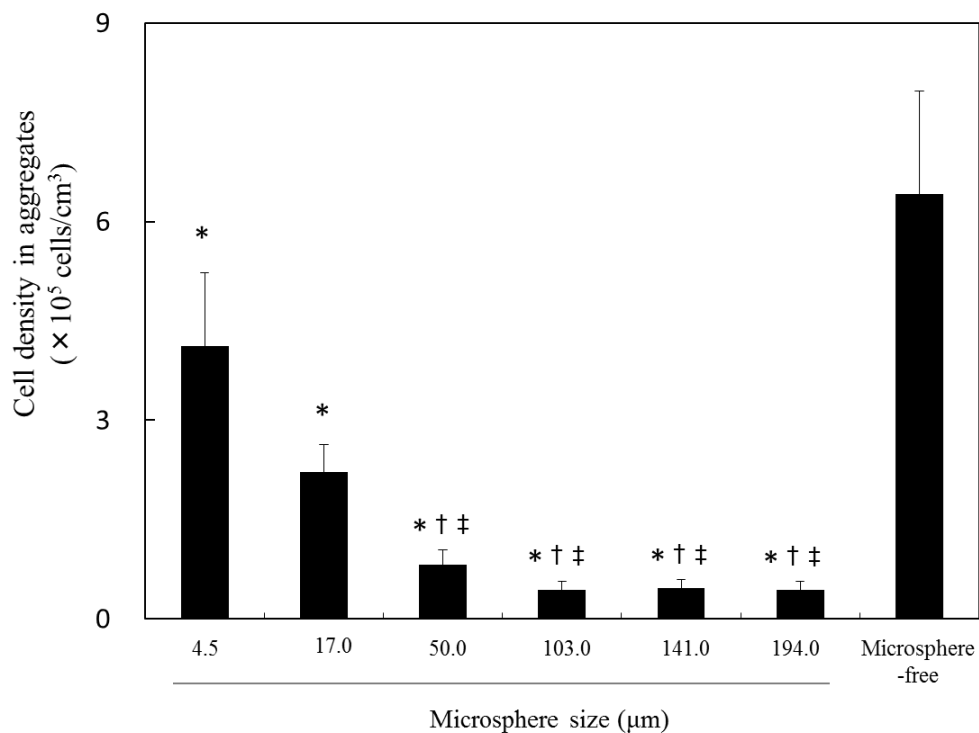


Figure 9. Cell density of MC3T3-E1 cell aggregates 21 days after incubation with or without GM. The cells were cultured at the same surface area of GM with sizes of 4.5, 17.0, 50.0, 103.0, 141.0, and 194.0 μm. \*,  $p < 0.05$ ; significant against the cell density of MC3T3-E1 cells in cell aggregates cultured without GM. †,  $p < 0.05$ ; significant against the cell density of MC3T3-E1 cells in cell aggregates cultured with GM sizes of 4.5 μm. ‡,  $p < 0.05$ ; significant against the cell density of MC3T3-E1 cells in cell aggregates cultured with GM sizes of 17.0 μm.

### 1.3.7 L-lactic acid/glucose ratio of cell aggregates incorporating gelatin hydrogel microspheres with different sizes

Figure 10 shows the L-lactic acid/glucose ratio of MC3T3-E1 cell in cell aggregates cultured with or without GM incorporation at the surface area of GM added ad cells cultured as the monolayers. Irrespective of the GM type, the L-lactic acid/glucose ratio of cell aggregates incorporating GM was significantly lower than that of GM-free cell aggregates. The L-lactic acid/glucose ratio became higher for cell aggregates incorporating GM as the GM became smaller. The lowest L-lactic acid/glucose ratio was observed for cell aggregates incorporating GM of larger size and cells of monolayer culture.

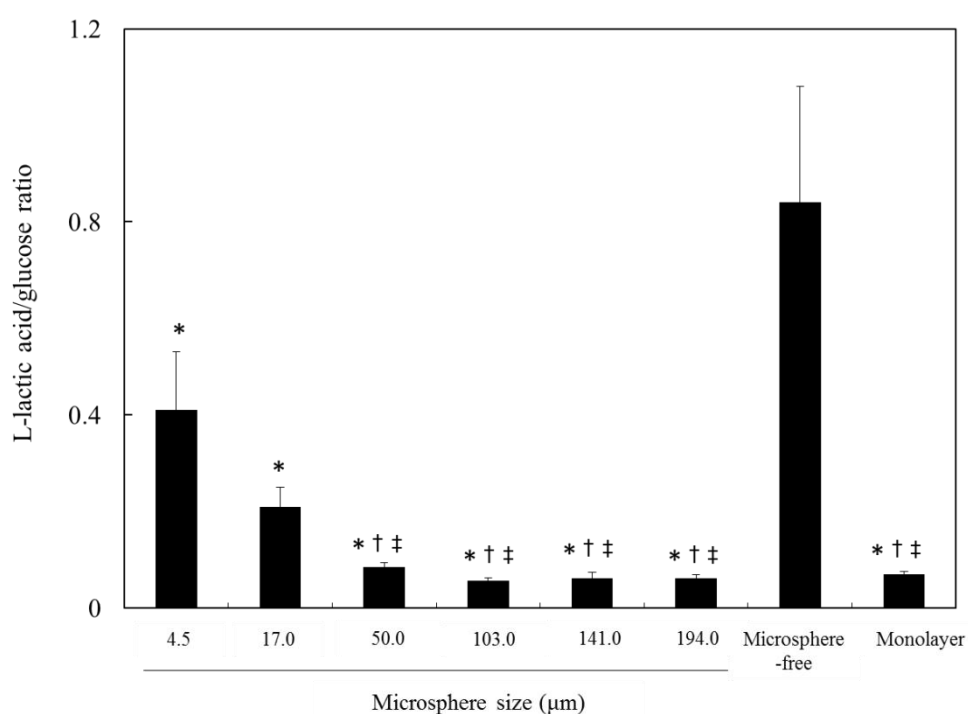


Figure 10. L-lactic acid/glucose ratio of MC3T3-E1 cells in cell aggregates 21 days after incubation with or without GM. The cells were cultured at

the same surface area of GM with sizes of 4.5, 17.0, 50.0, 103.0, 141.0, and 194.0  $\mu\text{m}$ . The cells were also as the monolayer culture without GM. \*,  $p < 0.05$ ; significant against the L-lactic acid/glucose ratio of MC3T3-E1 cells in cell aggregates cultured without GM. †,  $p < 0.05$ ; significant against the L-lactic acid/glucose ratio of MC3T3-E1 cells in cell aggregates cultured with GM sizes of 4.5  $\mu\text{m}$ . ‡,  $p < 0.05$ ; significant against the L-lactic acid/glucose ratio of MC3T3-E1 cells in cell aggregates cultured with GM sizes of 17.0  $\mu\text{m}$ .

### ***1.3.8 Alkaline Phosphatase activity and calcium content of cells in cell aggregates***

#### ***incorporating gelatin hydrogel microspheres with different sizes***

Figures 11 and 12 show the ALP activity and calcium content of MC3T3-E1 cell in cell aggregates cultured with or without GM incorporation at the same surface area of GM added. Little ALP activity was observed for GM-free cell aggregates. The ALP activity and calcium content tended to increase for cell aggregates incorporating GM as the GM size became longer. Both the values were significantly high compared with those of cells cultured in the 2D monolayer system. However, for cell aggregates incorporating 4.5  $\mu\text{m}$  GM, the ALP activity and calcium content were the same as those of GM-free aggregates.

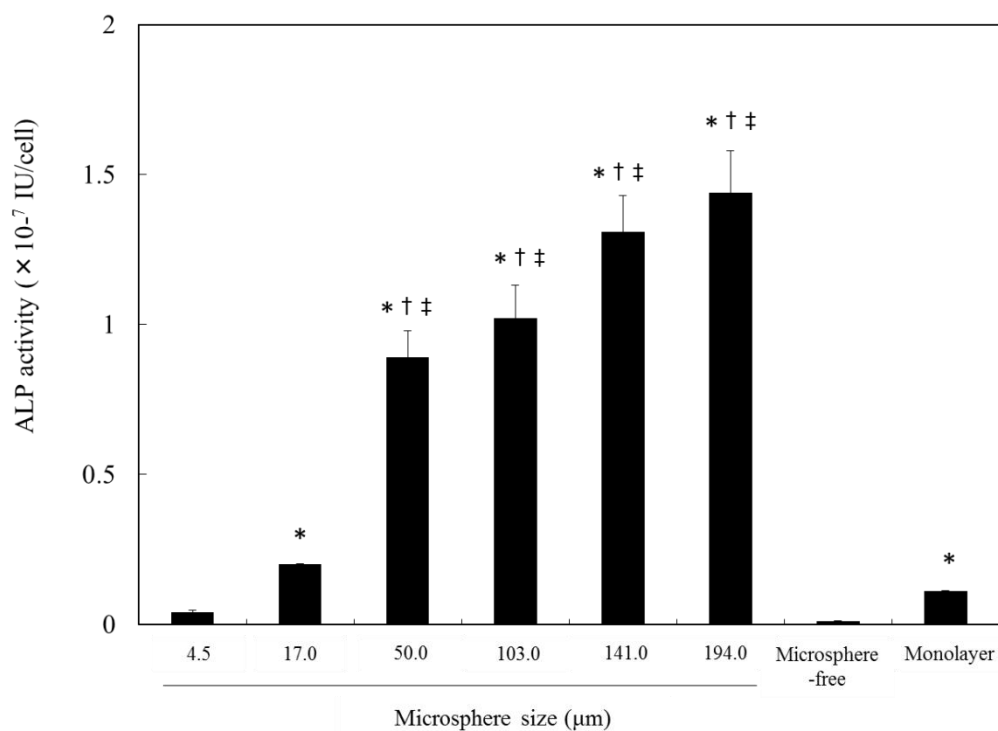


Figure 11. ALP activity of MC3T3-E1 cells in cell aggregates 21 days after incubation with or without GM. The cells were cultured at the same surface area of GM with sizes of 4.5, 17.0, 50.0, 103.0, 141.0, and 194.0 μm. The cells were also as the monolayer culture without GM. \*,  $p < 0.05$ ; significant against the ALP activity of MC3T3-E1 cells in cell aggregates cultured without GM. †,  $p < 0.05$ ; significant against the ALP activity of MC3T3-E1 cells in cell aggregates cultured with GM sizes of 4.5 μm. ‡,  $p < 0.05$ ; significant against the ALP activity of MC3T3-E1 cells in cell aggregates cultured with GM sizes of 17.0 μm.

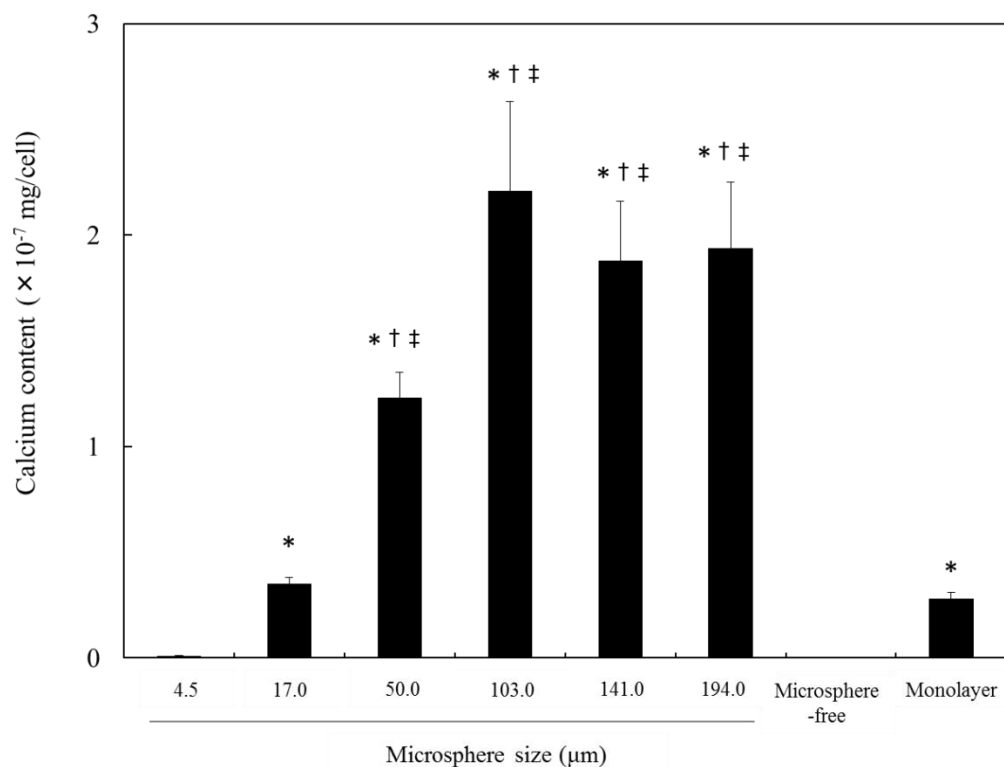


Figure 12. Calcium content of MC3T3-E1 cells in cell aggregates 21 days after incubation with or without GM. The cells were cultured at the same surface area of GM with sizes of 4.5, 17.0, 50.0, 103.0, 141.0, and 194.0 μm. The cells were also as the monolayer culture without GM. \*,  $p < 0.05$ ; significant against the calcium content of MC3T3-E1 cells in cell aggregates cultured without GM. †,  $p < 0.05$ ; significant against the calcium content of MC3T3-E1 cells in cell aggregates cultured with GM sizes of 4.5 μm. ‡,  $p < 0.05$ ; significant against the calcium content of MC3T3-E1 cells in cell aggregates cultured with GM sizes of 17.0 μm.

## 1.4 Discussion

Gelatin hydrogel microspheres (GM) with different sizes are used to improve the cells viability and functions in the aggregates. One research has been reported on the cell aggregates incorporating the microspheres of gelatin, poly (lactic-co-glycolic acid) (PLGA), and alginate [17]. Among the microspheres in this study, gelatin was selected as the microsphere material. Because the gelatin hydrogel microspheres have a cell adhesion ability better than PLGA and alginate microspheres. In addition, the oxygen and nutrients permeation through water phase of gelatin hydrogel microspheres is expected, in contrast to PLGA microspheres. Among the microsphere properties, the size of microspheres focused because the size is one of the important factors contributing to the oxygen and nutrients permeation for cell aggregates. There have been a few research papers to investigate the effect of microspheres sizes on the cells viability and functions in the aggregates [11, 14-21]. However, the size range of microspheres used was narrow and non-hydrogel type microspheres without the water phase, such as PLGA, were used.

In this study, GM were prepared by the dehydrothermal crosslinking, and then fractionated by sizes using sieves. The size of GM dispersed in water was different, but their degradability was similar (Figures 1 and 2). By using the well-designed GM, it is experimentally possible to evaluate the effect of GM size on the survival and functions of MC3T3-E1 cells aggregates incorporating GM.

The physicochemical parameters of GM, such as the diameter, volume, and surface area affect the preparation of MC3T3-E1 cell aggregates incorporating GM. MC3T3-E1 cell aggregates incorporating GM with different sizes were prepared under the following three conditions; the cell culture at the same number, volume, and surface

area of GM. The comparison studies at the same surface area of GM revealed that irrespective of the GM size, MC3T3-E1 cell aggregates incorporating GM were formed at approximately same incubation time point (Figure 5) and the time profile of live MC3T3-E1 cells number was similar (Figure 6C). However, when MC3T3-E1 cell aggregates incorporating GM were formed at the same number of GM added, the cell aggregates incorporating smaller sizes of GM were formed quickly (Figure 3). The number of MC3T3-E1 cells tended to increase for cell aggregates incorporating larger sizes of GM (Figure 6A). On the other hand, for MC3T3-E1 cell aggregates incorporating GM with different sizes, in the condition of same total volume of GM added, the aggregates formation became faster as the size of GM was larger (Figure 4). The number of MC3T3-E1 cells for cell aggregates tended to increase with a decrease in the GM size (Figure 6B). Considering that cells initially attached on the surface of GM and proliferated, it is conceivable that the surface area of GM is a key factor contributing to the cell attachment and the subsequent proliferation even although the GM size is different. The number of cells tended to increase before the MC3T3-E1 cell aggregates were completed to form. However, after the formation of MC3T3-E1 cell aggregates, the number of cells did not increase. The phenomenon may be explained in terms of the GM surface. After the attachment of MC3T3-E1 cells, the cells proliferated on the surface of GM, and then the formation of MC3T3-E1 cell aggregates would start. When MC3T3-E1 cell aggregates incorporating GM were formed at the same number of GM added, the surface area of GM in cell aggregates was larger for larger sizes of GM. As the result, the formation of cell aggregates incorporating GM would become slower. On the other hand, when MC3T3-E1 cell aggregates incorporating GM were prepared at the same volume of GM added, it is possible that the surface area of GM in cell

aggregates was smaller for larger sizes of GM, resulting in a rapid formation of cell aggregates incorporating GM. For the monolayer culture, the number of cells was similar or higher compared with that of cell aggregates incorporating GM. However, it is reported that cells present in the cell aggregates incorporating large number and size of GM showed faster proliferation than those of the monolayer culture [11]. This difference may be due to the ratio of cells to microspheres or the culture condition. As mentioned above, the large number and size of GM give cells larger surface area for cell proliferation, resulting in higher proliferation of cells in cell aggregates incorporating GM. The number of cells proliferated in cell aggregates incorporating GM largely depends on the experimental conditions.

The ratio of L-lactic acid production to glucose consumption is a general measure of aerobic metabolism [44]. The lower the ratio is, the higher the aerobic metabolism of cell aggregates is. It is apparent from Figure 10 that the lactic acid/glucose ratio of cell aggregates incorporating GM with larger sizes was significantly lower than that of aggregates without or with smaller sizes GM incorporation. The MC3T3-E1 cell aggregates incorporating the GM with larger sizes had low cell densities in the cell aggregates (Figures 8 and 9). Considering the formation of MC3T3-E1 cell aggregates incorporating GM at the same surface area of GM added, the volume of GM in cell aggregates was higher as the GM size became larger. Therefore, the size of cell aggregates incorporating GM prepared would be greatly influence by the volume of GM. Based on this, the lower cell density was observed for the cell aggregates incorporating larger sizes of GM. It is likely that the low cell density results in a lower ratio of L-lactic acid to glucose in an aerobic cell culture condition. In addition, the presence of GM with larger sizes homogeneously distributed in the cell aggregate may increase the

oxygen and nutrients permeation inside the aggregates. However, the lower number of live MC3T3-E1 cells inside MC3T3-E1 cell aggregates incorporating GM with smaller sizes was seen (Figure 7). The incorporation of GM with smaller sizes resulted in the higher lactic acid/glucose ratio of cell aggregates (Figure 11). Taken together, the incorporation of smaller GM would not be able to increase the oxygen level inside cell aggregates high enough not to die.

The ALP activity and calcium content, which are the measures of osteogenic differentiation of cell aggregates, were enhanced by the incorporation of GM, irrespective of the GM size (Figures 11 and 12). Some researches demonstrate that oxygen tension is an important mediator of osteogenic differentiation [46-52]. For MC3T3-E1 cell aggregates incorporating GM with larger size, it is possible that oxygen in the aggregate is at the normal level to allow cells to be alive even for long-term culture. On the contrary, MC3T3-E1 cell aggregates without or with smaller sizes GM incorporation showed lower levels of oxygen because of the restricted oxygen permeation. The homogeneously distributed presence of GM with large sizes in cell aggregates would keep the internal oxygen at the normal level, resulting in an enhanced osteogenic differentiation of MC3T3-E1 cells. It is conceivable that different sizes GM had different curvatures, which would affect the functions of MC3T3-E1 cells. It is apparent from Figure 8 that the shape of cells adhered on the GM surface was different (Figure 8). Titanium phosphate glass microspheres with larger sizes induced an enhanced proliferation and osteogenic differentiation of human mesenchymal stem cells [53]. It is reported that smaller sizes of gelatin-coated PLGA microspheres promoted to form an embryoid body with the microspheres incorporation and the consequent embryonic stem cell differentiation [18]. The difference of cell behavior between this

and reported studies can be explained by the preparation method of cell aggregates. The embryoid body was prepared by the co-culture of embryonic stem cells and gelatin-coated PLGA microspheres on a tissue culture dish with an orbital rotary shaker. Poor incorporation of gelatin-coated PLGA microspheres in the embryoid body formed was observed. The poor incorporation and distribution of gelatin-coated PLGA microspheres in the embryoid body caused poor embryonic stem cell differentiation. On the contrary, in this study, GM and cells were co-cultured in the non-cell adhesive, PVA-coated U-bottomed well of 96-multiwell plate. The PVA-coated U-bottomed well was effective in preparing the aggregation of GM and cells homogeneously mixed. This achieves higher incorporation and better distribution of GM in cell aggregates even in using the GM with larger size.

Better osteogenic differentiation of MC3T3-E1 cell aggregates incorporating GM with larger sizes than cells cultured in the monolayer system can be explained from the viewpoint of cell–cell interaction. In cell aggregates, cells interact in the 3D manner of natural cell–cell contact, whereas in the monolayer culture, the cells interact in a two-dimensional manner. We can say with certainty that the 3D interactions in the cell aggregates can give cells a more osteo-conductive condition.

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## **Chapter 2**

### **Preparation and functional evaluation of cell aggregates incorporating gelatin microspheres with different degradabilities**

#### **2.1 Introduction-**

Further development of basic cell researches is highly required to clarify the molecular mechanisms of cells proliferation and differentiation. Generally, the minimum unit of body is cell, but the functional unit is an aggregate of cells. In the body, most cells exist as an aggregate in which the physiological cell-cell interaction is formed and consequently enhances their survival and functions [1]. For example, embryonic stem cells generally aggregate to form an embryoid body and then initiate their differentiation into different cell lineages [2]. The aggregation of liver cells to form a spheroid is necessary to enhance the metabolic activity [3]. It is well known the cell aggregation generally produces extracellular matrix proteins more efficiently than single cells [4]. Looking at the structure of body tissues, such as liver and bone, the cell aggregates function as the minimum unit [5]. Taken together, it is possible that cell aggregation physiologically induces the cell-cell interactions, resulting in enhanced biological functions of cells.

The conventional cell culture has been performed in two-dimensional (2D) systems, which is quite different from the local environment of cells in living tissues. Therefore, three-dimensional (3D) cell culture technologies have been investigated [6-9]. However, there are some technological problems to be improved for the culture of cell aggregates. One of them is the death of cells in the center of aggregates with large size because of the lack of oxygen and nutrients supplies [10,11]. The cell death often makes it

practically difficult to continue the in vitro cell culture experiments over a long time period, although the long-term culture is required for basic researches of cell differentiation and organization.

To tackle this problem, we hypothesized that incorporation of hydrogel materials in the aggregate protected the aggregate from the lack of oxygen and nutrients because oxygen and nutrients can be permeated through the hydrogel matrix [12]. As the hydrogel material, in this study, gelatin hydrogel microspheres (GM) were selected. The previous study showed that the incorporation of GM protected the aggregate of rat bone marrow-derived mesenchymal stem cells (MSC) from the lack of nutrients and oxygen, resulting in enhanced MSC proliferation in aggregates [13].

Gelatin is a biodegradable biomaterial which has been extensively used for medical, pharmaceutical, and cosmetic applications. Its biosafety has been proven through the long-term practical usage [14]. Gelatin hydrogels of different shapes can be formulated, while their feasibility as cell culture substrates [15–17] and cell scaffolds for tissue regeneration [18–22] or as carriers of growth factors and drugs [23–30] has been experimentally demonstrated. GM can release growth factors to induce tissue regeneration [31–33]. Based on the availability and the easiness of hydrogel formulation, gelatin was selected as the hydrogel material.

There have been researches on the cell aggregates incorporating the microspheres of gelatin, poly (lactic-co-glycolic acid) (PLGA), and alginate [13, 34, 35, 36]. In this study, we have not used other types of microspheres to compare the cell functions. This is because the GM have better cell adhesion ability than PLGA and alginate microspheres. In addition, the GM have an ability to allow the controlled release of growth factors as reported previously [25]. The oxygen and nutrients permeation

through microspheres is expected, in contrast to PLGA microspheres.

The objective of this study is to investigate the effect of microspheres degradability on the cells viability and functions in the aggregates incorporating GM. Among the microsphere properties, the microsphere degradability is interesting and may affect the cell behavior in the aggregates. There is no research paper to investigate the effect of microspheres degradability on the cells viability and functions in the aggregates. GM with different degradabilities were prepared by changing the crosslinking conditions of gelatin. When incubated with different GM, the viability, metabolic activity, and osteogenic differentiation of MC3T3-E1 cells were evaluated in terms of microspheres degradability. We examine the degradation of GM in the cell aggregates to evaluate the dependence on the cell function.

## 2.2 Materials and methods

### ***2.2.1 Preparation of gelatin hydrogel microspheres with different degradabilities***

Gelatin hydrogel microspheres (GM) were prepared by chemical cross-linking of gelatin in a water-in-oil emulsion state according to the method previously reported [37]. Briefly, an aqueous solution (20 ml) of 10 wt% gelatin (isoelectric point = 5.0, weight-averaged molecular weight = 1,00,000, Nitta Gelatin Inc., Osaka, Japan) was preheated at 40 °C, and then added dropwise into 600 ml of olive oil (Wako Ltd, Osaka, Japan) at 40 °C, followed by stirring at 200 or 400 rpm. for 10 min to prepare a water-in-oil emulsion. The emulsion temperature was decreased to 4 °C for natural gelation of the gelatin solution to obtain non-crosslinked gelatin microspheres. The resulting microspheres were washed three times with cold acetone by centrifugation (5,000 rpm, 4 °C, 5 min) to completely exclude the residual oil. Then, they were fractionated by size using sieves with apertures of 20, 32, and 53 µm (Iida Seisakusyo Ltd, Osaka, Japan) and air dried at 4 °C. The non-crosslinked and dried gelatin microspheres (200 mg) with the sizes of 20-32 and 32-53 µm were treated in a vacuum oven at 140 °C to allow to dehydrothermally crosslink for gelatin for 6, 18, and 48 hr and 192 and 288 hr, respectively[26]. The photographs of gelatin hydrogel microspheres in the water-swollen state were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan). The size of 100 microspheres for each sample was measured using the computer program Image J (NIH Inc., Bethesda, USA) to calculate the average diameter.

### ***2.2.2 Evaluation of in vitro degradation of gelatin hydrogel microspheres with different degradabilities***

Dehydrothermally crosslinked GM (5 mg) was fully swollen in 750  $\mu$ l of double distilled water (DDW) for 1 hr at 37 °C in 2 ml tube. Next, 750  $\mu$ l of 2 M HCl was added and incubated at 37 °C for various time periods. Then, the tube was centrifuged at 5,000 rpm for 5 min at 37°C, followed by the collection of supernatant ( 200  $\mu$ l). After that, 200  $\mu$ l of 1 M HCl was added to continue incubation at 37 °C. To measure the amount of gelatin degraded, the adsorption of supernatant collected at 260 nm was measured by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech Inc., Cambridge, England).

### ***2.2.3 Cell culture experiments***

MC3T3-E1 cells of a pre-osteoblast line were cultured in alpha minimum essential medium ( $\alpha$ MEM) (Invitrogen Inc., Carlsbad, USA) supplemented with 10 vol % fetal calf serum (FCS) (Thermo Inc. Waltham, USA), penicillin (50 U/ml), and streptomycin (50 U/ml) (standard medium) and cultured at 37 °C in a 95 % air - 5 % carbon dioxide atmosphere. The culture medium was changed every 2 days and the confluent cells were subcultured through trypsinisation.

### ***2.2.4 Preparation of cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities***

A PVA sample (the degree of polymerization = 1,800 and the saponification = 88 mole %) kindly supplied from Unichika (Tokyo, Japan) was dissolved in phosphate-buffered solution (PBS) (1 wt%). The PVA solution was added to each well of flat- or round-bottomed (U-bottomed) 96-well culture plate ( 100  $\mu$ l/well) and incubated at 37 °C for 15 min. Then, the solution was removed by aspiration and the

wells were washed twice with PBS (100  $\mu$ l/well). Gelatin microspheres dehydrothermally crosslinked for 6, 18, 48, 192, and 288 hr and MC3T3-E1 cells were separately suspended in the standard medium. The suspension of GM (0 or  $2 \times 10^4$  microspheres/ml, 50  $\mu$ l/well) was added to the wells coated, followed by the addition of cell suspension ( $2 \times 10^4$  cells/ml, 50  $\mu$ l/well). Experiments for each sample were performed for three wells unless otherwise mentioned. The photographs of cell aggregates with or without GM were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan). The size of cell aggregates with or without GM measured using the computer program Image J (NIH Inc., Bethesda, USA) to calculate the average diameter.

#### ***2.2.5 Evaluation of cell distribution in cell aggregate incorporating gelatin hydrogel microspheres with different degradabilities***

MC3T3-E1 cells were cultured with GM dehydrothermally crosslinked for 48 hr to form the cell aggregates. After incubation for 14 days, the aggregates were fixed with 4 vol % paraformaldehyde at 4 °C for 1 hr and embedded in the optimal cutting temperature compound (Sakura Finetek Japan Ltd, Tokyo, Japan) and frozen using liquid nitrogen. The frozen samples were sectioned using a cryotome (CM3050S, Leica Microsystems, Wetzlar, Germany) and stained with TO-PRO-3 (Molecular Probes, Eu-gene, USA). The sections of 10  $\mu$ m thickness were viewed in a confocal laser scanning microscope (FV1000D, Olympus Ltd, Tokyo, Japan).

#### ***2.2.6 Evaluation of degradability of gelatin hydrogel microspheres in cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities***

MC3T3-E1 cells were cultured with GM in the similar way described above to form the cell aggregates. After culture for 7 and 21 days, the frozen sections of 10  $\mu\text{m}$  thickness were prepared and stained with eosin alcohol solution (Wako Ltd, Osaka, Japan), followed by viewing in microscope (CKX41, Olympus Ltd, Tokyo, Japan).

#### ***2.2.7 Measurement of cell density in cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities***

The cell density was calculated by following equation; cell density = the cell number in cell aggregate / cell aggregate volume ( $\text{cells}/\text{mm}^3$ ). The cell number in cell aggregate was determined by the fluorometric quantification of cellular DNA according to the method reported [38]. Briefly, the samples were lysed in 30 mM sodium citrate-buffered saline solution (SSC) (pH 7.4) containing 0.2 mg/ml sodium dodecylsulfate (SDS) at 37  $^{\circ}\text{C}$  for 12 hr with occasional mixing. The cell lysate (30  $\mu\text{l}$ ) was mixed with a dye solution (70  $\mu\text{l}$ ; 30 mM SSC, 10  $\mu\text{g}/\text{mL}$  Hoechst 33258 dye) and the fluorescent intensity of mixed solution was measured in a fluorescence spectrometer (F-2000, HITACHI Ltd, Tokyo, Japan) at the excitation and emission wavelengths of 355 and 460 nm, respectively. The calibration curve between the DNA and cell number was prepared by use of cells of known numbers.

#### ***2.2.8 Evaluation of cell viability in cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities***

Live/dead assays were conducted using Live/Dead<sup>®</sup> Viability/Cytotoxicity assay (Invitrogen Inc., UK) according to manufacture's protocol. The cell aggregates were rinsed with PBS 21 days later, and then incubated with mixed 2  $\mu\text{M}$  calcein AM and 4

$\mu$ M EthD-1 solution in PBS for 30 min at 37 °C in the dark, followed by observation through confocal laser scanning microscope (FV1000D, Olympus Ltd, Tokyo, Japan).

The number of live cells in MC3T3-E1 cell aggregates with or without GM was determined by counting the number of cell nuclei after crystal violet staining [36]. Briefly, a mixed solution of 0.2 M citric acid and 0.2 wt % crystal violet was added (100  $\mu$ l/well) to each well of 96 multi-well plate 4, 7, 14, and 21 days after MC3T3-E1 cell culture with or without GM dehydrothermally crosslinked. After crystal violet staining, the cells were lysed in 0.1 wt % Triton X-100 solution in PBS at 37 °C overnight to extract the nuclei from the cells. After pipetting, the nuclei collected were viewed under a microscope (CKX31-11PHP, Olympus Ltd, Tokyo, Japan) and counted in a hemocytometer (OneCell Inc., Hiroshima, Japan). The nuclei of live cells are generally round, while dead cell nuclei are irregularly shaped. The live cells can be distinguished from dead cells by their nucleus shape. Experiments for each sample were performed for three wells for each sample unless mentioned otherwise.

### ***2.2.9 Measurement of L-lactic acid/glucose ratio of cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities***

The amount of glucose consumed by MC3T3-E1 cell aggregates formed was determined by measuring the change in glucose concentration in the culture medium using a Glutest Neo Super test kit (Sanwa kagaku kenkyusyo Co. Ltd, Japan) 4, 7, 14, and 21 days after incubation. After the similar time intervals, the amount of L-lactic acid produced by MC3T3-E1 cell aggregates was determined with an E-kit (R-Biopharm AG Co. Ltd, Germany). The number of live cells was determined by the crystal violet staining as described above and used to normalize the amount of glucose

consumption and L-lactic acid produced to the number of live cells. The L-lactic acid/glucose ratio was calculated as a measure of cells metabolic activity [39].

#### ***2.2.10 Evaluation of Osteogenic differentiation of cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities***

MC3T3-E1 cells were cultured with GM similarly form the cell aggregates. As a control, MC3T3-E1 cells were cultured in the flat-bottomed wells of 96-well culture plates at a density of  $1 \times 10^3$  cells per well. The cell aggregates were cultured for 21 days in the osteogenic differentiation medium of  $\alpha$ MEM containing 10 mM  $\beta$ -glycerophosphate, 0.2 mM L-ascorbic acid 2-phosphate (Sigma inc., Poole, UK), 10 nM dexamethasone, and 10 vol% fetal bovine serum.

The alkaline phosphatase (ALP) activity of MC3T3-E1 cells was determined using a Lab Assay ALP kit (Wako Ltd, Osaka, Japan) according to the procedure previously reported [40, 41]. For the calcium assay, the sample was incubated in 1M HCl solution for 4 hr at 4°C. The calcium amount of HCl solution was determined using a calcium E-HA kit (Wako Ltd, Osaka, Japan) according to manufacturer's protocol. The cell aggregates with or without gelatin microspheres dehydrothermally crosslinked for 192 hr at 140 °C cultured for 21 days in the osteogenic differentiation medium  $\alpha$ MEM. Then, preparation of MC3T3-E1 cell frozen sections was performed similarly. Frozen section samples were stained in 40 mM Alizarin Red S (pH4.2) for 10 min at room temperature.

#### ***2.2.11 Statistical analysis***

All the statistical data are expressed as the mean $\pm$ standard error of the mean (SEM).

The data were analyzed by student t-test and the statistical significance was accepted at the p value of  $<0.05$ .

## 2.3 Results

### 2.3.1 Characterization of gelatin hydrogel microspheres

Figure 1 shows the microscopic images of gelatin hydrogel microspheres (GM). The microspheres were of spherical shape and had a smooth surface. The size of the GM in the swollen condition was similar, irrespective of their crosslinking time. The frame of microspheres crosslinked for 6 hr was unclear, because of the high swelling in water. Then, to evaluate the in vitro degradability of GM, degradation tests of GM in HCl solution were performed.

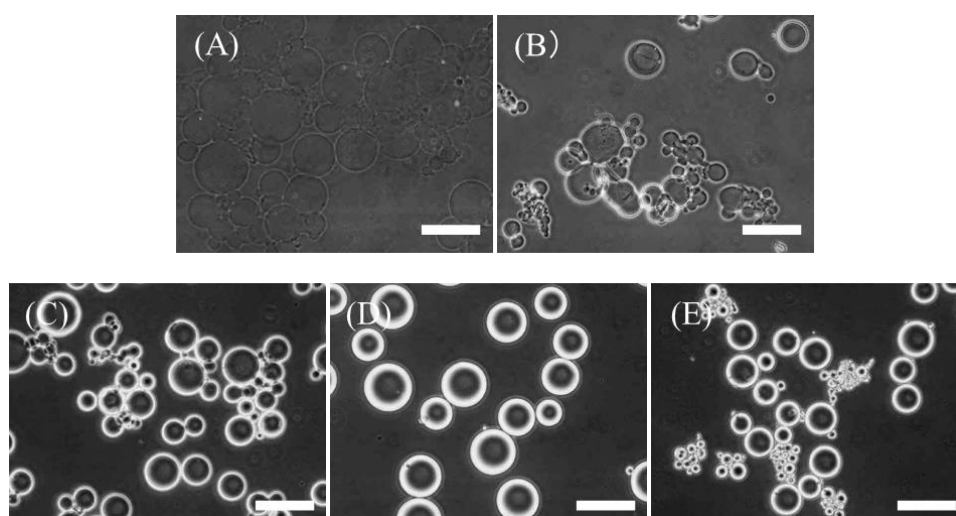


Figure 1. Microscopic pictures of GM dispersed in water. The microspheres dehydrothermally crosslinked for 6 (A), 18 (B), 48 (C), 192 (D), and 288 hr (E) at 140 °C. Scale bar is 100  $\mu\text{m}$  length.

Figure 2 shows the in vitro degradation profiles of GM dehydrothermally crosslinked. The degradation time of GM increased with an increase in the crosslinking time for

microspheres preparation.

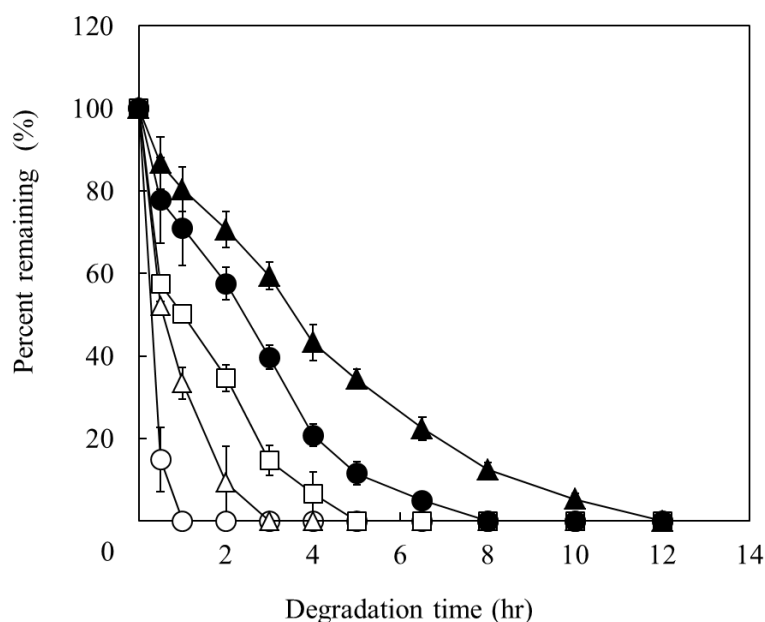


Figure 2. In vitro degradation profiles of GM dehydrothermally crosslinked for 6 (○), 18 (△), 48 (□), 192 (●), and 288 hr (▲) at 140 °C.

### 2.3.2 Formation of cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities

Figure 3 shows the microscopic pictures of MC3T3-E1 cell aggregates 1, 3, 5, 7, 14, and 21 days after incubation with or without GM. MC3T3-E1 cell aggregates were formed only for the U-bottomed well, in contrast to the flat-bottomed one (data not shown).

The size of cell aggregates tended to decrease with incubation time when the GM dehydrothermally crosslinked for 6, 18, and 48 hr. On the contrary, the size did not

change for other GM. The size of cell aggregates was larger for slow-degraded GM. In the microspheres-free culture, the size was smaller and did not change over the time studied.

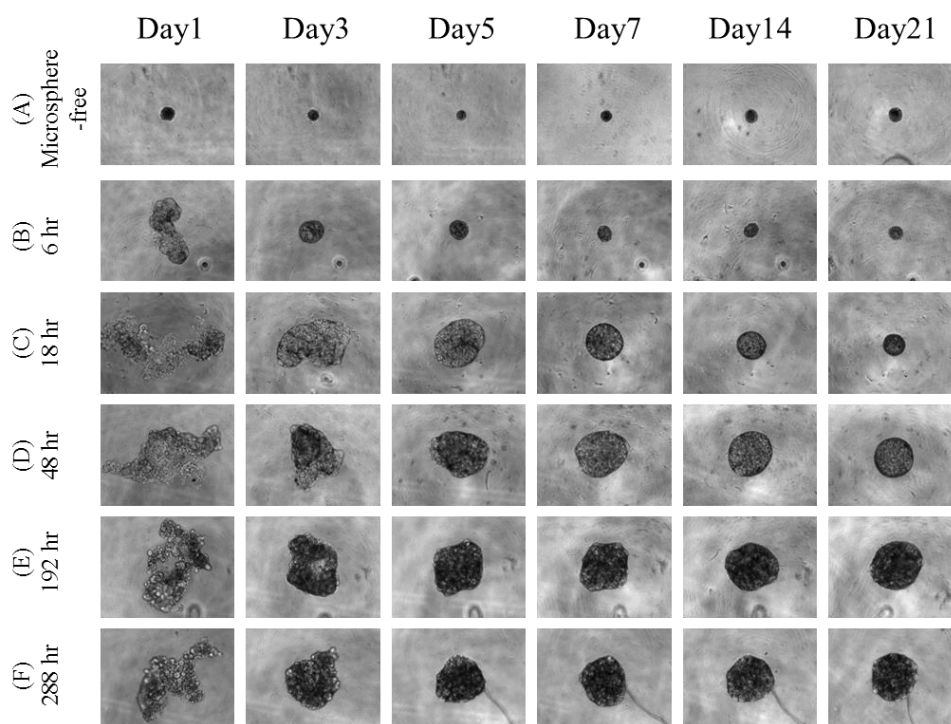


Figure 3. Microscopic pictures of MC3T3-E1 cell aggregates 1, 3, 5, 7, 14, and 21 days after incubation with (A-E) or without GM (F). MC3T3-E1 cells were cultured with GM dehydrothermally crosslinked for 6 (A), 18 (B), 48 (C), 192 (D), and 288 hr (E) at 140 °C. Scale bar is 500  $\mu$ m length.

### 2.3.3 Distribution of cells in cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities

Figure 4 shows the microscopic pictures of MC3T3-E1 cell aggregates 14 days after incubation with GM. The MC3T3-E1 cells and GM seemed to be distributed

homogeneously in the cell aggregate. Similar conditions were observed for GM with different degradabilities (data not shown).

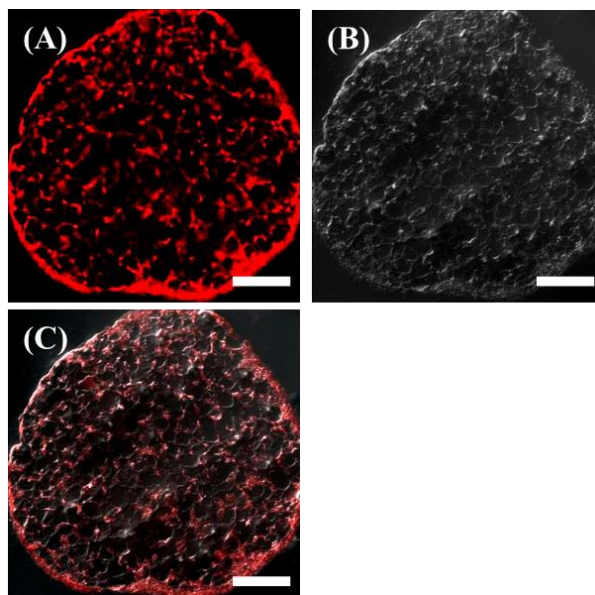


Figure 4. Microscopic pictures of MC3T3-E1 cell aggregates 14 days after incubation with GM dehydrothermally crosslinked for 48 hr at 140°C: (A) A confocal microscopic picture of a cell aggregate stained with the TO-PRO-3, (B) A light microscopic image of the corresponding cell aggregate, and (C) A superposed image of (A) and (B). The scale bar is 100  $\mu\text{m}$  length.

#### ***2.3.4 Degradability of gelatin hydrogel microspheres in cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities***

Figure 5 shows the microscopic pictures of MC3T3-E1 cell aggregates 7 and 21 days after incubation with or without eosin-stained GM. For the slow-degraded GM, large

amount of GM in MC3T3-E1 cell aggregates was observed 21 days after incubation. Few microspheres were detected in the cell aggregates for faster-degraded GM.

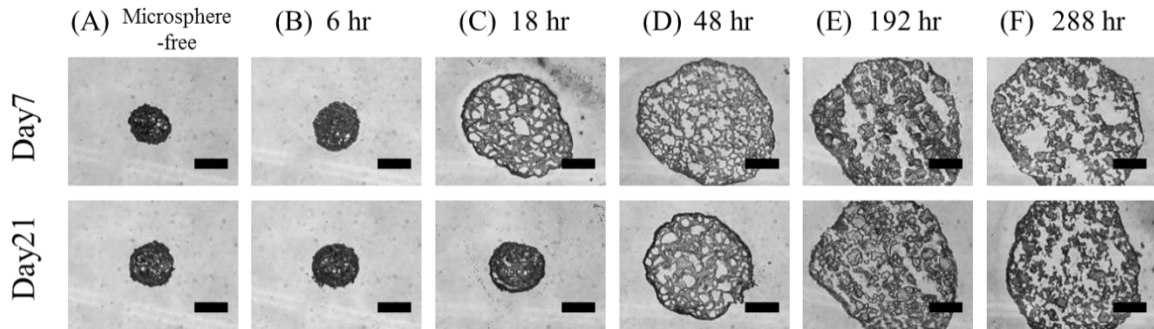


Figure 5. Microscopic pictures of MC3T3-E1 cell aggregates 7 and 21 days after incubation with (A-E) or without GM stained with eosin (F). MC3T3-E1 cells were cultured with GM dehydrothermally crosslinked for 6 (A), 18 (B), 48 (C), 192 (D), and 288 hr (E) at 140 °C. Scale bar is 100  $\mu\text{m}$  length.

### ***2.3.5 Cell conditions in cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities***

Figure 6 shows the live/dead assay of MC3T3-E1 cell aggregates 21 days after incubation. For microspheres prepared at crosslinking times of 6 and 18 hr, similarly to the microspheres-free culture, the cells in center of cell aggregates were dead. However, larger numbers of cells in the MC3T3-E1 cell aggregates survived for GM dehydrothermally crosslinked for 48, 192, and 288 hr at 140°C. Then, to evaluate the number of live cells in cell aggregates, cell nuclei were stained by crystal violet and the number of cell nuclei were measured.

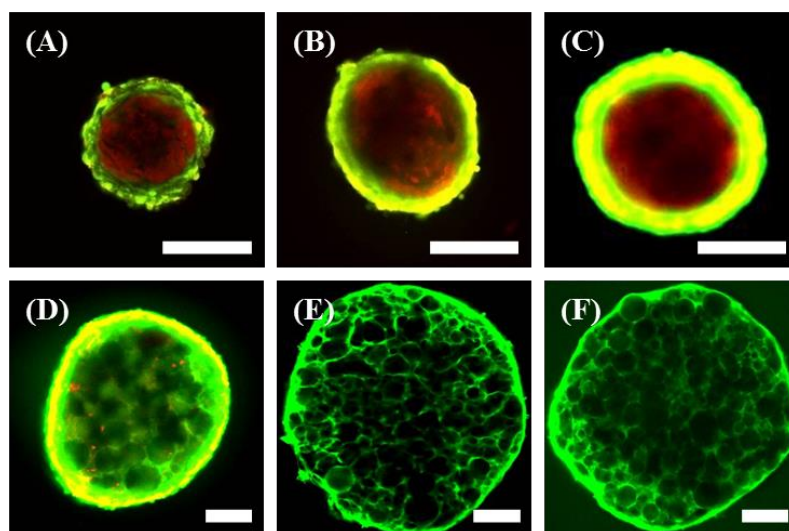


Figure 6. Live/dead assay of MC3T3-E1 cell aggregates 21 days after incubation with (A-E) or without GM (F). MC3T3-E1 cells were cultured with GM dehydrothermally crosslinked for 6 (A), 18 (B), 48 (C), 192 (D), and 288 hr (E) at 140 °C. Scale bar is 100  $\mu$ m length.

Figure 7 shows the number of live MC3T3-E1 cells 4, 7, 14, and 21 days after incubation with or without GM. The number of live cells in MC3T3-E1 cell aggregates without GM did not increase. In contrast, the number of live cells in MC3T3-E1 cell aggregates with GM increased up to a certain level. Larger numbers of live cells were observed for the incubation with slow-degraded GM.

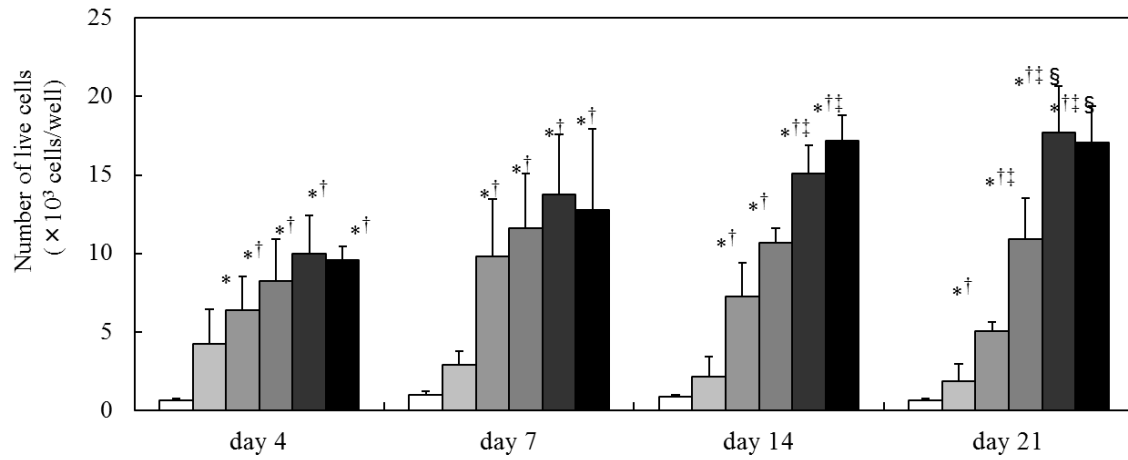


Figure 7. The number of live MC3T3-E1 cells 4, 7, 14, and 21 days after incubation with or without GM (■). MC3T3-E1 cells were cultured with GM dehydrothermally crosslinked for 6 (■), 18 (■), 48 (■), 192 (■), and 288 (■) hr at 140 °C. \*,  $p < 0.05$ ; significant against the number of live MC3T3-E1 cells in cell aggregate at the corresponding time. †,  $p < 0.05$ ; significant against the number of live MC3T3-E1 cells incubated with GM dehydrothermally crosslinked for 6 hr at 140 °C at the corresponding time. ‡,  $p < 0.05$ ; significant against the number of live MC3T3-E1 cells incubated with GM dehydrothermally crosslinked for 18 hr at 140 °C at the corresponding time. §,  $p < 0.05$ ; significant against the number of live MC3T3-E1 cells incubated with GM dehydrothermally crosslinked for 48 hr at 140 °C at the corresponding time.

### 2.3.6 L-lactic acid/glucose ratio of cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities

Figure 8 shows the L-lactic acid/glucose ratio of MC3T3-E1 cell in cell aggregates

cultured with or without GM. Irrespective of the microspheres type, the L-lactic acid/glucose ratio of MC3T3-E1 cell aggregates with GM was lower than that of without GM. The L-lactic acid/glucose ratio became lower for slow-degraded and was similar to that of monolayer cell cultures.

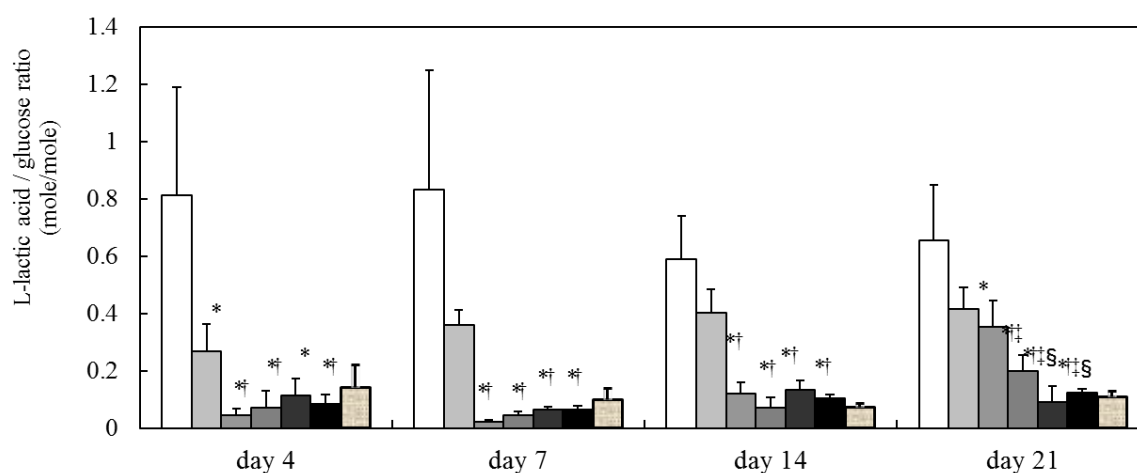


Figure 8. The L-lactic acid/glucose ratio of MC3T3-E1 cell in cell aggregates 4, 7, 14, and 21 days after incubation with or without GM (■). MC3T3-E1 cells were cultured with GM dehydrothermally crosslinked for 6 (■), 18 (■), 48 (■), 192 (■), and 288 (■) hr at 140 °C. The cells were cultured in each well of flat-bottomed 96-well plates as a monolayer culture without GM (■). \*,  $p < 0.05$ ; significant against the L-lactic acid/glucose ratio MC3T3-E1 cell in cell aggregates cultured without GM at the corresponding time. †,  $p < 0.05$ ; significant against the L-lactic acid/glucose ratio of MC3T3-E1 cell in cell aggregates cultured with GM dehydrothermally crosslinked for 6 hr at 140 °C at the corresponding time. ‡,  $p < 0.05$ ; significant against the L-lactic acid/glucose ratio of MC3T3-E1

cell in cell aggregates cultured with gelatin microspheres dehydrothermally crosslinked for 18 hr at 140 °C at the corresponding time. <sup>§</sup>, p<0.05; significant against the L-lactic acid/glucose ratio of MC3T3-E1 cell in cell aggregates cultured with GM dehydrothermally crosslinked for 48 hr at 140 °C at the corresponding time.

### ***2.3.7 Alkaline Phosphatase activity and calcium content of cell aggregates incorporating gelatin hydrogel microspheres with different degradabilities***

Figure 9A shows the ALP activity of MC3T3-E1 cell in cell aggregates cultured with or without GM. No ALP activity was observed for MC3T3-E1 cell aggregates without GM. The higher ALP activity was observed for MC3T3-E1 cell aggregates with slow-degraded GM crosslinked for 192 and 288 hr, and the ALP activity was significantly higher than that of monolayer cell cultures.

Figure 9B shows the calcium content of MC3T3-E1 cell in cell aggregates cultured with or without GM. No calcium was observed for MC3T3-E1 cell aggregates without GM. The greater calcium content was observed for MC3T3-E1 cell aggregates with slow-degraded GM, and the calcium content was significantly higher than that of monolayer cell cultures. Figures 9C and D show the microscopic pictures of MC3T3-E1 cell aggregates with or without alizarin red-stained GM 21 days after culture. The homogeneous calcium deposition was observed for MC3T3-E1 cell aggregates cultured with GM whereas no deposition was done for microspheres-free culture.

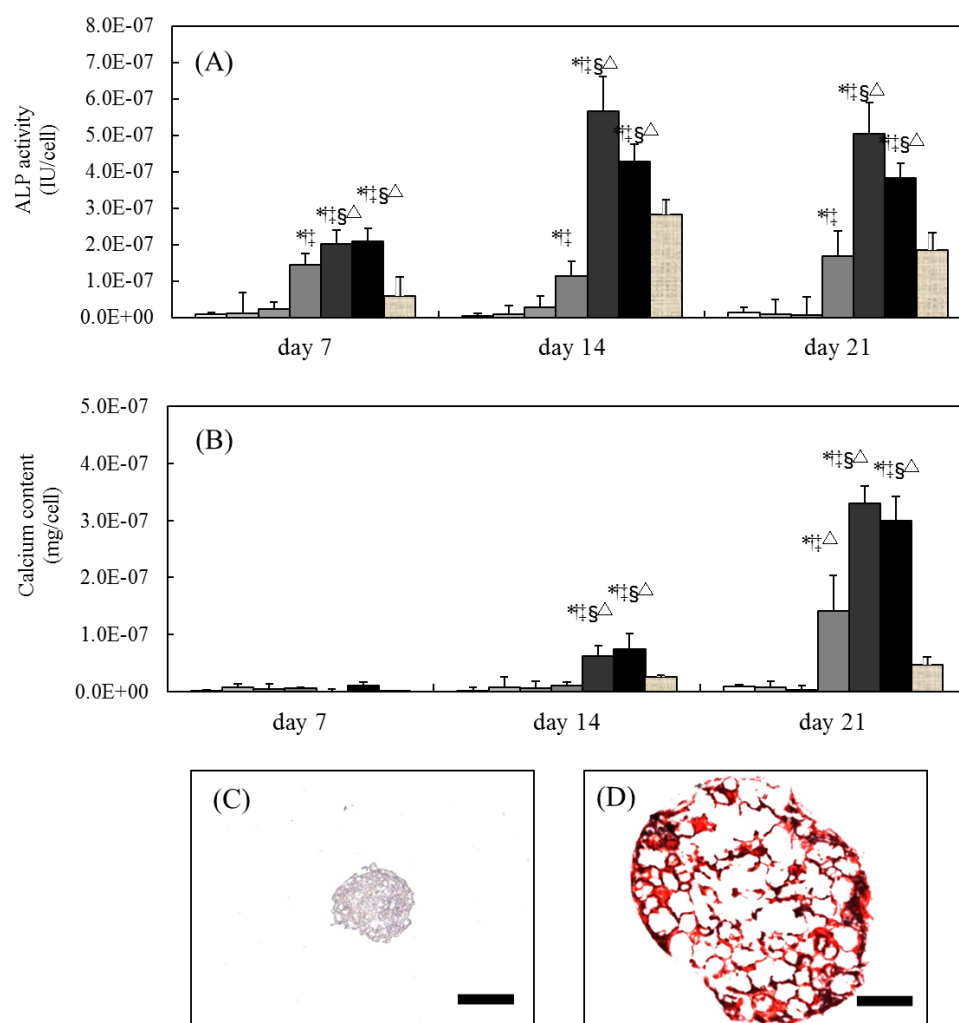


Figure 9. The osteogenic differentiation MC3T3-E1 cells in cell aggregates 7, 14, and 21 days after incubation with or without GM(■). MC3T3-E1 cells were cultured with gelatin microsphere dehydrothermally crosslinked for 6 (■), 18 (■), 48 (■), 192 (■), and 288 (■)hr at 140 °C. The cells were cultured in each well of flat-bottomed 96-well plates as a monolayer culture without GM (■). (A) The ALP activity of MC3T3-E1 cell in cell aggregates cultured with or without GM and MC3T3-E1 cells cultured as a monolayer. \*,  $p < 0.05$ ; significant against the ALP activity MC3T3-E1 cells

in aggregates cultured without GM at the corresponding time. <sup>†</sup>,  $p < 0.05$ ; significant against the ALP activity of MC3T3-E1 cells in aggregates cultured with GM dehydrothermally crosslinked for 6 hr at 140 °C at the corresponding time. <sup>‡</sup>,  $p < 0.05$ ; significant against the ALP activity of MC3T3-E1 cells in aggregates cultured with GM dehydrothermally crosslinked for 18 hr at 140 °C at the corresponding time. <sup>§</sup>,  $p < 0.05$ ; significant against the ALP activity of MC3T3-E1 cells in aggregates cultured with GM dehydrothermally crosslinked for 48 hr at 140 °C at the corresponding time. <sup>Δ</sup>,  $p < 0.05$ ; significant against the ALP activity of MC3T3-E1 monolayer cultured at the corresponding time. (B) The calcium content of MC3T3-E1 cell aggregates cultured with or without GM and MC3T3-E1 cells cultured as a monolayer. <sup>\*</sup>,  $p < 0.05$ ; significant against the calcium content MC3T3-E1 cells in aggregates cultured without GM at the corresponding time. <sup>†</sup>,  $p < 0.05$ ; significant against the calcium content of MC3T3-E1 cells in aggregates cultured with GM dehydrothermally crosslinked for 6 hr at 140 °C at the corresponding time. <sup>‡</sup>,  $p < 0.05$ ; significant against the calcium content of MC3T3-E1 cells in aggregates cultured with GM dehydrothermally crosslinked for 18 hr at 140 °C at the corresponding time. <sup>§</sup>,  $p < 0.05$ ; significant against the calcium content of MC3T3-E1 cells in aggregates cultured with GM dehydrothermally crosslinked for 48 hr at 140 °C at the corresponding time. <sup>Δ</sup>,  $p < 0.05$ ; significant against the calcium content of MC3T3-E1 monolayer cultured at the corresponding time (C) A microscopic picture of MC3T3-E1 cell aggregates without GM cultured for 21 days stained with alizarin red. (D)

A microscopic picture of MC3T3-E1 cell aggregates with GM dehydrothermally crosslinked for 192 hr at 140 °C stained with alizarin red. The scale bar is 100  $\mu\text{m}$  length.

## **2.4 Discussion**

The present study demonstrates that the incorporation of gelatin hydrogel microspheres gave the cells inside the aggregates a better condition to proliferate well and differentiate into bone cells. Chung and Park also claimed that mass transport of oxygen and nutrients to the cells inside the cell aggregates could be enhanced cell viability and differentiation [35]. The proliferation and differentiation extents depended on the type of microspheres incorporated. The slower the degradation of GM in the aggregates, the better the survival or metabolic and differentiation functions improved. This can be explained in terms of gelatin microspheres degradability. It is possible that the longer remaining of microspheres in the aggregates due to their slower degradation gives the cells in the aggregate better supply of oxygen and nutrient, resulting in enhancing the number of live cells and osteogenic differentiation.

In this study, the GM with similar sizes were prepared by different time periods of crosslinking (Figure 1). The frame of GM crosslinked for 6 hr was unclear, because of the high swelling in water. The in vitro degradation tests showed that the degradation time of GM in HCl solution increase with an increased in the crosslinking time (Figure 2). This is because the crosslinking extent of GM increased with an increase in the crosslinking time, resulting in their slower degradation.

The cell aggregates were formed only for the U-bottomed well, in contrast to the flat-bottomed one (data not shown). It is conceivable that an U-shaped bottom well allows cells to accumulate in the center of well. As a result, the frequency of cell–cell contact increases, resulting in the better formation of cell aggregates. On the other hand, in flat-bottomed wells, such a cell accumulation does not occur and cell aggregates form randomly and heterogeneously and the efficiency was very low [42]. This is due to less

frequent cell–cell contact. Although cells do not adhere to the surface of PVA-coated substrates, it is possible that less frequent cell–cell contact on the flat substrate does not result in efficient formation of cell aggregates.

To confirm the degradability of GM in MC3T3-E1 cell aggregates, the frozen sections of MC3T3-E1 cell aggregates 7 and 21 days after incubation with eosin-stained GM were prepared (Figure 5). Larger amount of GM in MC3T3-E1 cell aggregates were observed for slow-degraded GM. The degradability order of GM in MC3T3-E1 cell aggregates was similar to that of degradability in HCl solution. The size of cell aggregates tended to decrease with incubation time when fast-degraded GM were used. On the contrary, the size did not change for slow-degraded GM (Figures 3). This can be explained in terms of GM degradability and cytocompatibility. Since the GM show similar compatibility to cells, cells proliferate on their surface and form the aggregate incorporating microspheres similarly at the initial stage of the cell culture. However, the degradability of GM is different. Slow-degraded GM remained in MC3T3-E1 cell aggregates for longer time period. The cells would proliferate well in the cell aggregates because the microspheres of the cell scaffold are present and the oxygen and nutrients conditions are better. However, the cell proliferation was limited (Figure 7) and the size did not change (Figures 3 and 5). The reason is not clear at present. It is highly conceivable that the supply and demand of oxygen and nutrients for cells are well balanced, resulting in maintained survived of the cells in the aggregates.

The ALP activity and calcium content of cell aggregates, a measure of osteogenic differentiation, was enhanced (Figure 9A and 9B). In addition, it is recognized that the oxygen tension is one of the important mediators of osteogenic differentiation [43-46]. For MC3T3-E1 cell aggregates incorporating slow-degraded GM, the oxygen level in

the aggregate would keep at the normal level during the long-term culture. In contrast, MC3T3-E1 cell aggregates with or without GM had different situations. The disappear of microspheres due to their fast degradation would reduce the oxygen level. The homogeneous distribution of GM in cell aggregates would keep the internal oxygen at the normal, resulting in the higher viability and osteogenic differentiation of MC3T3-E1 cells.

Better osteogenic differentiation of 3D cell aggregates than the monolayer culture has previously been reported [47]. A larger number of genes, such as ALP, bone morphogenic protein 2 (BMP2), and osteopontin, showed up-regulation after induction of osteogenic differentiation in 3D MSC aggregates, compared with those of cells monolayer cultured. It is demonstrated that the surrounding condition of cells through cell-cell interactions affects the cell behavior. In this study, MC3T3-E1 cell aggregates incorporating slow-degraded GM showed higher osteogenic differentiation compared with the cells monolayer cultured. It is conceivable that in addition to the oxygen supply to cells, the 3D cell-cell interaction gave cells more conducive to osteogenic differentiation. The detailed investigation on the mechanism of osteogenic differentiation should be required. In future, osteogenic differentiation in aggregates with or without GM will be investigated further by histological, biochemical, and immunohistochemical examination.

One of the merits to form cell aggregates is to enhance the cellular functions compared with the cells seeded onto the surface of scaffolds because of the formation of 3-dimensional cell-cell interaction.

Recently, the approach of cell transplantation and tissue regeneration with cell aggregates is reported to demonstrate the therapeutic efficacy superior to that of single

cells [35, 48]. The incorporation of growth factors into the GM may enhance the cell functions of aggregates, while it is possible that cell aggregates are improve the efficacy of cell therapy.

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## **Chapter 3**

### **Preparation of cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities**

#### **3.1 Introduction**

Recently, three-dimensional (3D) cell culture technologies have been increasing noticed [1–4]. The conventional cell culture has been performed in two-dimensional (2D) systems, which is quite different from the local environment of cells in living tissues. Looking at the structure of body tissues, such as liver and bone, cell aggregates physiologically work as the minimum unit of function [5]. In the 3D cell culture, it is possible that the cell aggregation permits efficient cell–cell interactions, resulting in an enhanced biological functions of cells. For example, embryonic stem cells generally aggregate to form an embryoid body, and consequently initiate differentiation into different cell lineages [6]. The aggregation of liver cells to form a spheroid is necessary to enhance their metabolic activity [7]. In addition, cell aggregates produce extracellular matrix proteins more efficiently than single cells [8]. However, there are some problems for the culture of cell aggregates. One of them is that when the cell aggregate grows up, cells in the center of large aggregates die because of the lack of oxygen and nutrients supplies [9,10]. The previous study revealed that the incorporation of slow-degraded GM prevents the mouse preosteoblast MC3T3-E1 cells aggregated from a lack of oxygen in the long-term culture, resulting in enhanced cell proliferation and osteogenic differentiation in MC3T3-E1 aggregates [11]. The number of papers about the 3D cell aggregates culture system combined with microspheres has been increased recently [12–16]. However, there are few research reports to promote and control the functions of

cells in cell aggregates by making use of microspheres.

Gelatin is a biodegradable biomaterial which has been extensively used for medical, pharmaceutical, and cosmetic applications. Its biosafety has been proven through the long-term practical usage [17]. Hydrogels of different shapes can be formulated and their feasibility as cell culture substrates [18–20] and cell scaffolds for tissue regeneration [21–25] and as the release carriers of growth factors and drugs [26–33] has been experimentally demonstrated. Gelatin microspheres are effective in enhancing growth factor-induced tissue regeneration [34–36]. Based on their biological availability and the easiness of hydrogel formulation, gelatin was selected as the hydrogel material in this study. BMP2 is recognized as one of the strong osteogenic factors [37–39] and can be released by the gelatin hydrogel microspheres in a controlled manner [25, 40–42]. The time profile of BMP2 is controllable by changing the degradability of gelatin hydrogel microspheres containing BMP2 [17].

The objective of this study is to evaluate the survival and functions of cells in cell aggregates with or without the incorporation of GM. In this study, GM with different degradabilities were prepared, followed by the incorporation of bone morphogenetic protein (BMP)-2 to obtain GM containing BMP2 (GM-BMP2). Then, cell aggregates incorporating the GM-BMP2 with different degradabilities were prepared to evaluate the viability, L-lactic acid/glucose ratio, and osteogenic differentiation of cell aggregates comparing with free BMP2 addition.

## 3.2 Materials and methods

### ***3.2.1 Preparation of gelatin hydrogel microspheres with different degradabilities***

Gelatin hydrogel microspheres (GM) were prepared by the chemical cross-linking of gelatin in a water-in-oil emulsion state according to the method previously reported [43]. Briefly, an aqueous solution (20 ml) of 10 wt% gelatin (isoelectric point 9.0, weight-averaged molecular weight 1,00,000, Nitta Gelatin Inc., Osaka, Japan) was preheated at 40 °C, and then added dropwise into 600 ml of olive oil (Wako Ltd, Osaka, Japan) at 40 °C, followed by stirring at 150 or 400 rpm. for 10 min to prepare a water-in-oil emulsion. The emulsion temperature was decreased to 4 °C for the natural gelation of gelatin solution to obtain non-crosslinked microspheres. The resulting GM were washed three times with cold acetone in combination with centrifugation (5000 rpm., 4 °C, 5 min) to completely exclude the residual oil. Then, they were fractionated by size using sieves with apertures of 20, 32, and 53 µm (Iida Seisakusyo Ltd, Osaka, Japan) and air dried at 4 °C. The non-crosslinked and dried GM (200 mg) were treated in a vacuum oven at 140 °C and 0.1 Torr for the dehydrothermal crosslinking of gelatin for 18, 48, 96, and 192 hr according to the method previously reported [10]. The pictures of GM in the water swollen state were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan). The size of 100 microspheres for each sample was measured using the computer program Image J (NIH inc., Bethesda, USA) to calculate the average diameter.

### ***3.2.2 Evaluation of in vitro degradation of gelatin hydrogel microspheres with different degradabilities***

GM dehydrothermally crosslinked (5mg) was fully swollen in a tube (Thermo Inc., Walsam, USA) with 750  $\mu$ l of double distilled water (DDW) for 1 hr at 37 °C. To the suspension of GM swollen, 750  $\mu$ l of 2 M HCl was added, followed by incubation at 37 °C for determined time periods. Then, the tube was centrifuged at 5000 rpm, 37 °C for 5 min and 200  $\mu$ l of supernatant was sampled. After that, 200  $\mu$ l of 1 M HCl was added and incubation at 37 °C was continued until to next sampling time. To measure the amount of gelatin degraded by HCl, the adsorption of supernatant collected at 260 nm was measured by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech inc., Cambridge, England). Experiments were performed for 3 samples independently unless otherwise mentioned.

### ***3.2.3 Preparation of gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities***

Human recombinant BMP2 was kindly supplied by Astellas Pharma Inc. (Tokyo, Japan). BMP2 was dissolved in DDW to give a solution at BMP2 concentrations of 1, 10, 100, 1000, and 10000  $\mu$ g/ml. The BMP2 solution (20  $\mu$ l) was dropped onto 2 mg of freeze-dried GM, followed by leaving at 37 °C for 1 hr for the impregnation of BMP2 into the GM to prepare GM containing BMP2 (GM-BMP2). The BMP2 solution was completely absorbed into the microspheres through the impregnation process because the solution volume was much less than that theoretically required for the equilibrated swelling of GM.

### ***3.2.4 Cell culture experiments***

MC3T3-E1 preosteoblasts were cultured in alpha minimum essential medium

( $\alpha$ MEM) (Invitrogen inc., Carlsbad, USA) supplemented with 10 vol% fetal calf serum (FCS) (Thermo Inc., Waltham, USA), penicillin (50 U/ml), and streptomycin (50 U/ml) (standard medium) at 37 °C in a 95% air- 5% carbon dioxide atmosphere. The culture medium was changed every 2 days and confluent cells were subcultured through trypsinisation.

### ***3.2.5 Preparation of cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities***

Poly (vinyl alcohol) (PVA, 1800 degree of polymerization and 88 mole% saponification) kindly supplied from Unichika Ltd (Tokyo, Japan) was dissolved in phosphate-buffered saline solution (PBS, pH7.4) (1 wt%). This solution (100  $\mu$ l/well) was added to each well of a 96-multiwell culture plate with round-bottomed (U-bottomed) wells and incubated at 37 °C for 15 min. Then, the solution was removed by aspiration and the wells were washed twice with PBS (100  $\mu$ l/well). GM-BMP2 dehydrothermally crosslinked for 18, 48, 96, and 192 hr and MC3T3-E1 cells were separately suspended in the standard medium. The suspension of GM (0,  $2 \times 10^4$ , 50  $\mu$ l/well) was added to the PVA-treated wells, followed by the addition of MC3T3-E1 suspension ( $2 \times 10^5$  cells/ml, 50  $\mu$ l /well). Experiments of each sample were performed for 3 wells unless mentioned otherwise. The pictures of cell aggregates with or without GM-BMP2 incorporation were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan). The size of cell aggregates was measured using the computer program Image J (NIH inc., Bethesda, USA) to calculate the average diameter.

### ***3.2.6 Osteogenic differentiation of MC3T3-E1 cells in cell aggregates cultured with***

***gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities***

MC3T3-E1 cells were cultured with GM and GM-BMP2 in the similar way described above to form the cell aggregates. The cell aggregates were cultured for 7 or 21 days in osteogenic differentiation medium  $\alpha$ MEM containing 10 mM  $\beta$ -glycerophosphate, 0.2 mM L-ascorbic acid 2-phosphate (Sigma inc., Poole, UK), 10 nM dexamethasone, and 10 vol% fetal bovine serum. To evaluate the effect of BMP2 on the cell differentiation, BMP2 was added to the cell culture in the following three ways; the presence of GM-BMP2 containing 1, 10, 100, 1000, or 10000 ng, 1, 10, 100, 1000 and 10000 ng of free BMP2, or 10 times addition of free BMP2 2, 4, 6, 8, 10, 12, 14, 17 and, 20 days after incubation (Figure 1).

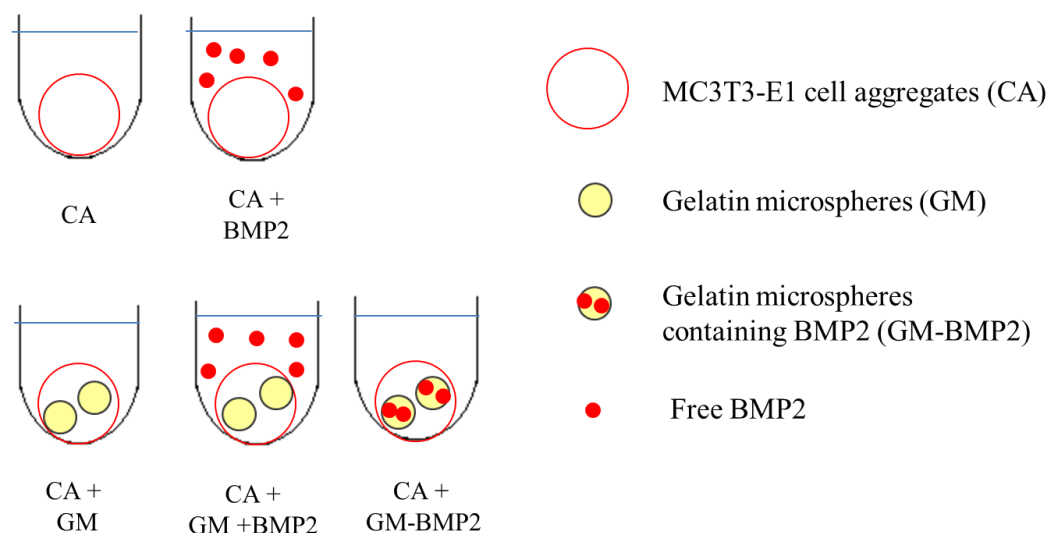


Figure 1. Preparation condition of MC3T3-E1 cell aggregates without or with GM incorporation. MC3T3-E1 cells were incubated with or without GM containing endogenous BMP2 (GM-BMP2) or once or 10 times

addition of free BMP2.

### ***3.2.7 Evaluation of BMP2 distribution in cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2***

BMP2 was labelled by Alexa Fluor® 594 Protein Labeling Kit (Thermo Inc., Waltham, USA) according to the manufacturer's instructions. MC3T3-E1 cells were cultured with gelatin microspheres incorporating Alexa594-labelled BMP2 dehydrothermally crosslinked for 18, 48, and 192 hr to form the cell aggregates. After incubation for 7 days, the aggregates were fixed with 4 vol% paraformaldehyde at 4 °C for 1 hr and embedded in an optimal cutting temperature compound (Sakura Finetek Japan Co. Ltd, Tokyo, Japan) and frozen by liquid nitrogen. The frozen samples were sectioned using a cryotome (CM3050S, Leica Microsystems, Wetzlar, Germany), to prepare the sections of 10 µm thickness to view in a confocal laser scanning microscope (FV1000D, Olympus Ltd, Tokyo, Japan).

### ***3.2.8 Measurement of live cells number in cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities***

The number of live cells in MC3T3-E1 cell aggregates without or with GM-BMP2 was determined by counting the number of cells nuclei after their crystal violet staining [44]. Briefly, a mixed solution of 0.2 M citric acid and 0.2 wt% crystal violet was added (100 µl/well) to each well of 96-multiwell culture plate 7 and 21 days after MC3T3-E1 cell culture without or with GM-BMP2 dehydrothermally crosslinked for 18, 48, 96, and 192 hr at 140 °C. After crystal violet staining, the cells were lysed in 0.1 wt%

Triton X-100 in PBS at 37 °C overnight to prepare the cell nuclei. After pipetting, the nuclei collected were viewed under a microscope (CKX31-11PHP, Olympus Ltd, Tokyo, Japan) and counted in a hemocytometer (OneCell Inc., Hiroshima, Japan). The nuclei of live cells are generally round, while those of dead cells are irregularly shaped. Thus, live cells can be distinguished from dead cells by their nucleus shape. Experiments of each sample were performed for 3 wells for each sample unless mentioned otherwise.

***3.2.9 Measurement of L-lactic acid/glucose ratio of cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities***

The similar culture of MC3T3-E1 cells with GM and GM-BMP2 was performed to form the cell aggregates. The amount of glucose consumed by MC3T3-E1 cell aggregates was determined by measuring the change in glucose concentration in the culture medium using a Glutest Neo Super test kit (Sanwa kagaku kenkyusyo Co. Ltd, Japan) 7 and 21 days after incubation. Similarly, the amount of L-lactic acid produced by MC3T3-E1 cell aggregates was determined with an E-kit (R-Biopharm AG Co. Ltd, Germany) 7 and 21 days after incubation. The number of live cells was determined by the crystal violet staining as described above and normalized by the amount of glucose consumption and L-lactic acid produced to the number of live cells. The L-lactic acid/glucose ratio was calculated as a measure of the metabolic activity of cells [45]. Experiments of each sample were performed for 3 wells unless mentioned otherwise.

***3.2.10 Evaluation of osteogenic differentiation of cell aggregates cultured with***

***gelatin hydrogel microspheres containing bone morphogenic protein-2***

The messenger ribonucleic acid (mRNA) expression of runt-related transcription factor 2 (RUNX2) was evaluated by a real-time polymerase chain reaction (PCR). Briefly, The RNA was extracted and cleaned up using the Qiagen RNeasy® Plus Mini kit (Qiagen Ltd, Hilden, Germany) according to the manufacturer's instructions. The total RNA sample was reverse-transcribed to cDNA using SuperScript VILO™ (Invitrogen inc., Carlsbad, CA). Then, the resulting cDNA was subjected to real-time PCR by using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems inc., Carlsbad, CA) to determine the gene expression of RUNX2. The PCR reaction was carried out with specific primers in the presence of Power SYBR Green (Applied Biosystems inc., Carlsbad, CA) as described in the manufacturer's instruction. The level of gene expression was normalized by that of the housekeeping gene,  $\beta$ -actin, and calculated as the ratio to that of MC3T3-E1 cells cultured without GM. Experiments were performed for 4 specimens independently for each sample. The primer sequences for RUNX2 and  $\beta$ -actin were as follows, RUNX2:

5-TCCACAAGGACAGAGTCAGATTAC-3, 5- TGGCTCAGATAGGAGGGGTA-3;  
 $\beta$ -actin: 5- AACAGTCCGCCTAGAAGCAC-3, 5-CGTTGACATCCGTAAAGACC-3.

The alkaline phosphatase (ALP) activity of MC3T3-E1 cells were determined using a Lab Assay ALP kit (Wako Ltd, Osaka, Japan) according to the procedure previously reported [46]. The sample was incubated in 1M HCl solution for 24 hr at 4 °C. The calcium amount of HCl solution was determined using a calcium E-HA kit (Wako Ltd, Osaka, Japan) according to the manufacture's protocol. Experiments of each sample were performed for 3 wells unless otherwise mentioned.

### ***3.2.11 Statistical analysis***

All the statistical data are expressed as the mean $\pm$ standard error of the mean (SEM). The data were analyzed by t-test to determine the statistical significance of differences between two mean values, which was accepted at p value of  $<0.05$ .

### 3.3 Results

#### 3.3.1 Sizes of gelatin hydrogel microspheres

Figure 2 shows the typical microscopic pictures of GM. The GM were of spherical shape and had a smooth surface. The size of GM prepared dehydrothermally crosslinked for 18, 48, 96, and 192 hr at 140 °C in the swollen condition was  $56.1 \pm 17.6$ ,  $50.7 \pm 18.4$ ,  $46.8 \pm 19.3$ , and  $55.1 \pm 18.8$   $\mu\text{m}$ , respectively.

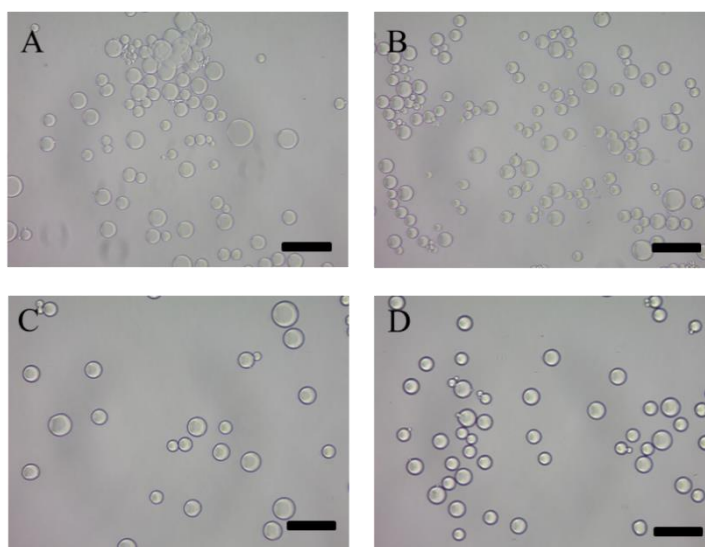


Figure 2. Microscopic pictures of gelatin hydrogel microspheres (GM) dispersed in water. The GM were dehydrothermally crosslinked for 18 (A), 48 (B), 96 (C), and 192 hr (D) at 140 °C. Scale bar. 100  $\mu\text{m}$ .

Figure 3 shows the in vitro degradation profiles of GM dehydrothermally crosslinked for 18, 48, 96, and 192 hr at 140 °C. As the dehydrothermal crosslinking time was longer, the degradation of GM became slower.

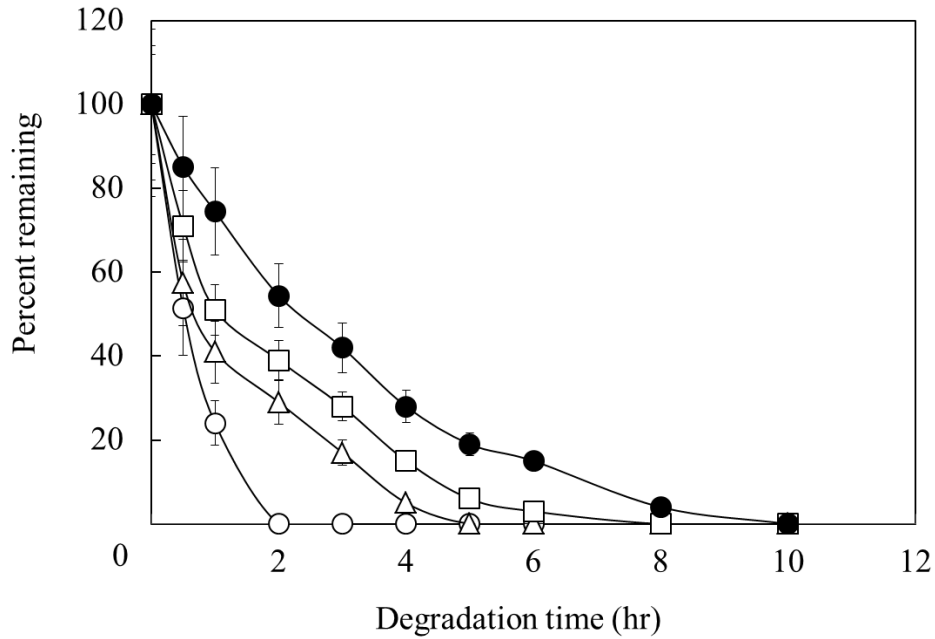


Figure 3. In vitro degradation profiles of GM dehydrothermally crosslinked for 18 (○), 48 (△), 96 (□) and 192 hr (●) at 140 °C.

### 3.3.2 Formation of Cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2

Figure 4 shows the microscopic pictures of MC3T3-E1 cell aggregates 2, 7, 14, and 21 days after incubation without or with GM. Irrespective of the GM type, MC3T3-E1 cell aggregates were formed in the U-bottomed well culture dish. With the incubation period, the size of MC3T3-E1 cell aggregates tended to become smaller, although the size change depended on the type of GM used. After 21 days culture, few GM remained in MC3T3-E1 cell aggregates for GM dehydrothermally crosslinked for 18 hr.

MC3T3-E1 cell aggregates of larger size were observed for GM of slower degradation which were crosslinked for longer time periods.

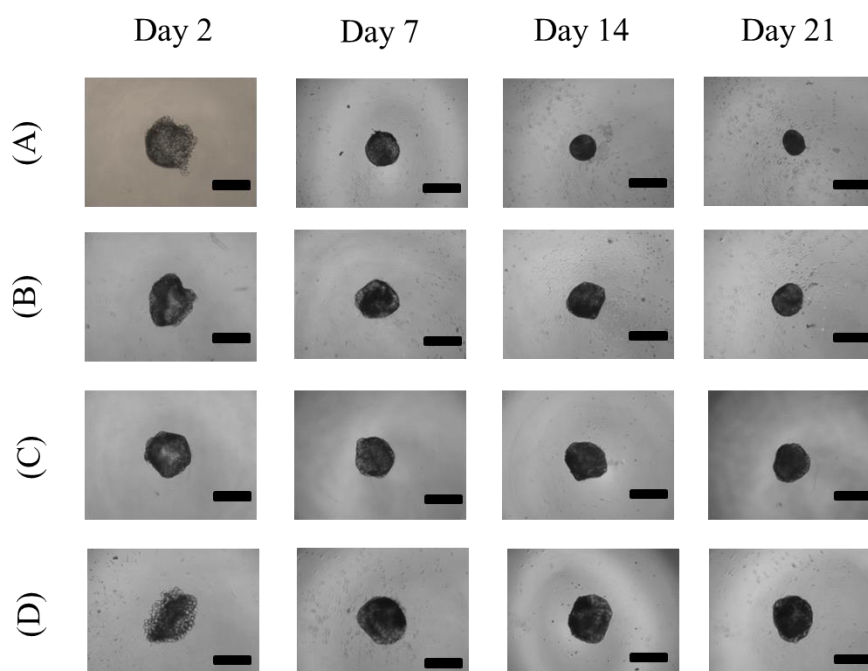


Figure 4. Light microscopic pictures of MC3T3-E1 cell aggregates 2, 7, 14, and 21 days after incubation with GM dehydrothermally crosslinked for 18 (A), 48 (B), 96 (C), and 192 hr (D) at 140 °C. Scale bar. 500  $\mu$  m.

### ***3.3.3 BMP2 distribution in cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2***

Figure 5 shows the microscopic pictures of MC3T3-E1 cell aggregates 7 days after incubation with GM-BMP2 crosslinked for 18, 48, and 192 hr or 10 times addition of free BMP2. The BMP2 was distributed homogeneously in the MC3T3-E1 cell aggregates incubated with GM-BMP2, irrespective of the GM-BMP2 type. On the other hand, a heterogeneous distribution of BMP2 was observed for free BMP2. BMP2 tended to be distributed on the peripheral portion of cell aggregates.

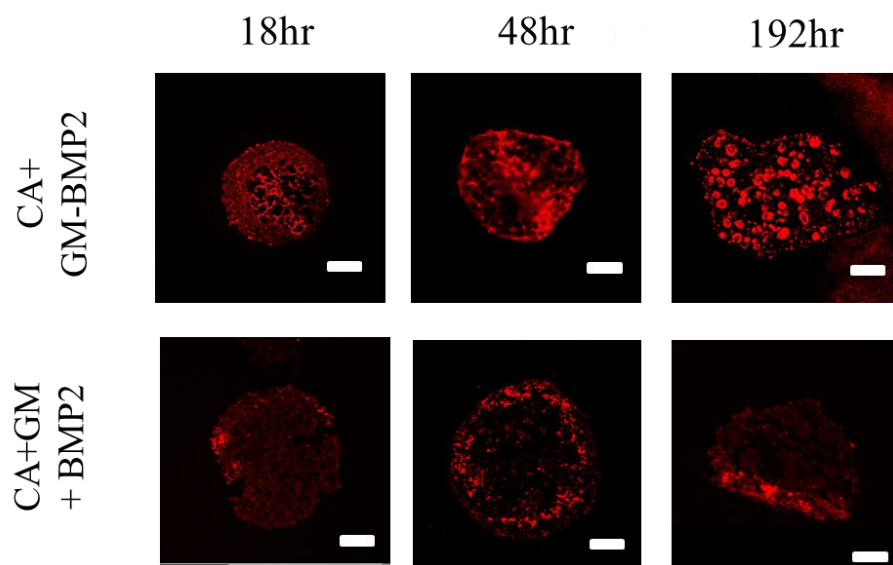


Figure 5. Fluorescent microscopic pictures of MC3T3-E1 cells aggregates 7 days after incubation with GM containing Alexa594-labeled BMP2 or 10 times addition of free BMP2. GM were dehydrothermally crosslinked for 18, 48, and 192 hr at 140 °C. Scale bar. 100  $\mu$ m.

### 3.3.4 Live cell number and L-lactic acid/glucose ratio of cells in cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2

Figure 6 shows the number of live MC3T3-E1 cells 7 and 21 days after incubation without or with GM-BMP2 and GM plus free BMP2. Without GM, the number of live cells in MC3T3-E1 cell aggregates did not increase in the presence or absence of BMP2. On the contrary, the number of live cells in MC3T3-E1 cell aggregates cultured with GM-BMP2 tended to increase with incubation time. Larger number of live cells was observed for the GM of slower degradation.

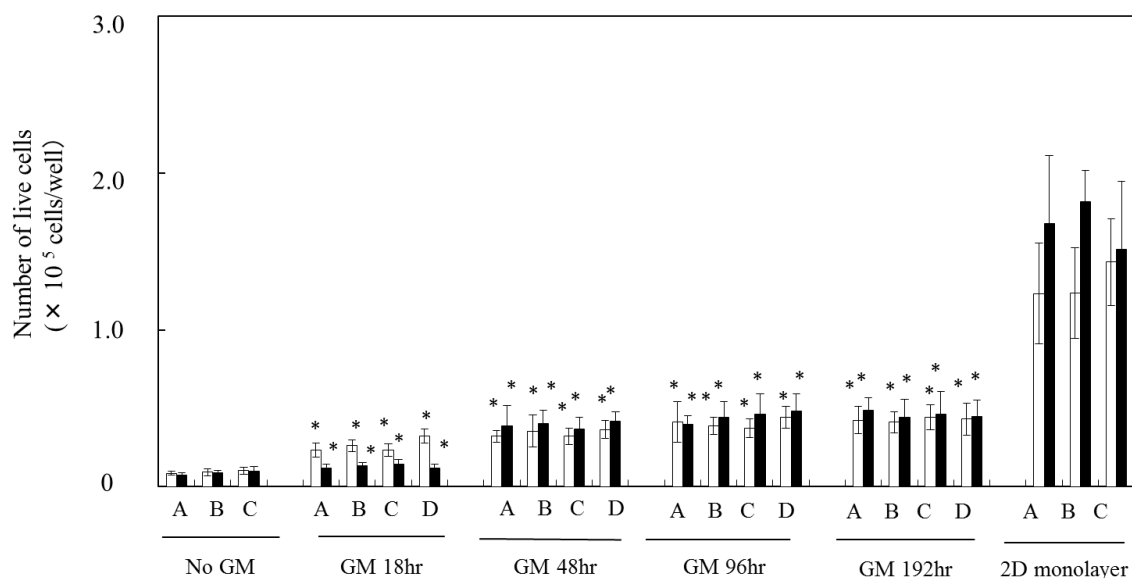


Figure 6. The number of live MC3T3-E1 cells in aggregates without or with GM incorporation 7 (□) and 21 days (■) after incubation in the absence (A) or presence of once (B) or 10 times (C) addition of free BMP2 or GM-BMP2 (D). The total amount of BMP2 added was 10 ng. GM were dehydrothermally crosslinked for 18, 48, 96, and 192 hr at 140 °C. The MC3T3-E1 cells without GM were cultured in each well of flat-bottomed 96-multiwell plates as a 2D monolayer culture in the absence (A) or presence of once (B) or 10 times (C) addition of free BMP2 into the medium. \*  $p < 0.05$ , significant against the number of live MC3T3-E1 cells in MC3T3-E1 cell aggregates cultured without GM at the corresponding time point.

Figure 7 shows the L-lactic acid/glucose ratio of MC3T3-E1 cell in cell aggregates cultured without or with GM-BMP2 and GM plus free BMP2 and MC3T3-E1 cells

cultured as the monolayer. Irrespective of the BMP2 presence, the L-lactic acid/glucose ratio of MC3T3-E1 cell aggregates cultured with GM-BMP2 was significantly lower than that of cell aggregates without GM. The lower L-lactic acid/glucose ratio was observed for GM-BMP2 of slower degradation. The ratio of MC3T3-E1 cell aggregates cultured with GM-BMP2 crosslinked for 48, 96, and 192 hr was similar to that of monolayer cell culture.

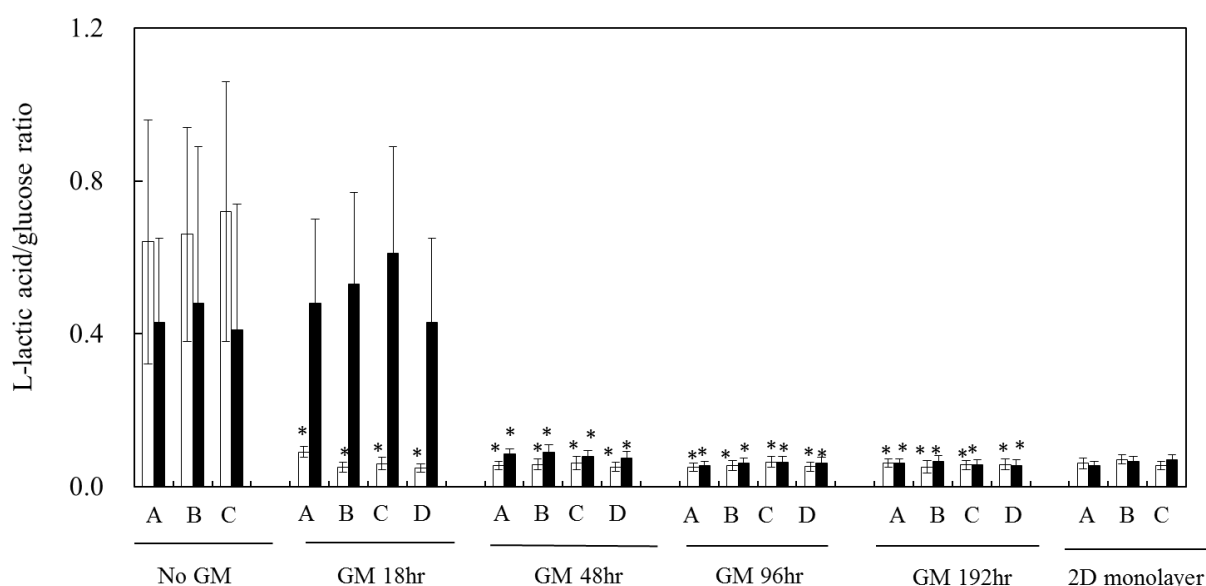


Figure 7. The L-lactic acid/glucose ratio of MC3T3-E1 cells in aggregates without or with GM incorporation 7 (□) and 21 days (■) after incubation in the absence (A) or presence of once (B) or 10 times (C) addition of free BMP2 or GM-BMP2 (D). The total amount of BMP2 added was 10 ng. GM were dehydrothermally crosslinked for 18, 48, 96, and 192 hr at 140 °C. The MC3T3-E1 cells without GM were cultured in each well of flat-bottomed 96-multiwell plates as a 2D monolayer culture in the absence

(A) or presence of once (B) or 10 times (C) addition of free BMP2 into the medium. \*  $p < 0.05$ , significant against the lactic acid/glucose ratio of MC3T3-E1 cells in MC3T3-E1 cell aggregates cultured without GM at the corresponding time point.

### ***3.3.5 Alkaline Phosphatase activity and Runt-related transcription factor 2 mRNA expression of cells in cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2***

Figures 8 and 9 shows the ALP activity and RUNX2 mRNA expression of MC3T3-E1 cell in cell aggregates cultured with GM-BMP2 at different BMP2 concentrations. The significantly higher ALP activity was observed for MC3T3-E1 cell aggregates cultured with GM-BMP2 than those of once or 10 times addition of BMP2 at BMP2 concentrations other than 1ng/ml. The RUNX2 expression for the GM-BMP2 absence culture was significantly high compared with that of free BMP2 culture.

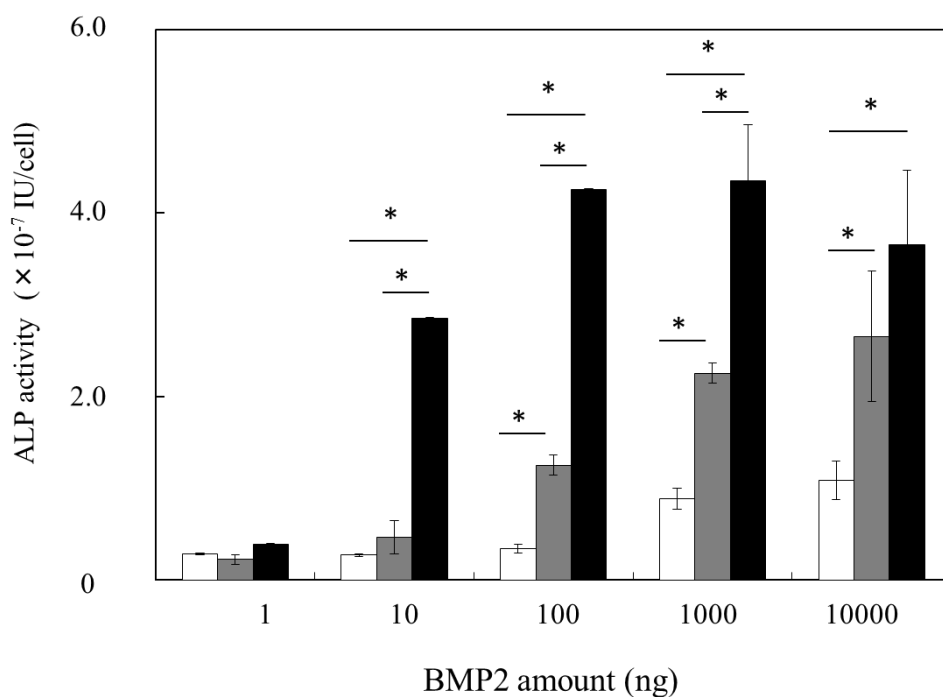


Figure 8. The ALP activity of MC3T3-E1 cells in aggregates 7 days after incubation with once (□) or 10 times (■) addition of free BMP2 or GM-BMP2 (■). The total amount of BMP2 added were 1, 10, 100, 1000, and 10000 ng. GM were dehydrothermally crosslinked for 18 hr at 140 °C.

\*  $p < 0.05$ , significant against the ALP activity between two groups.

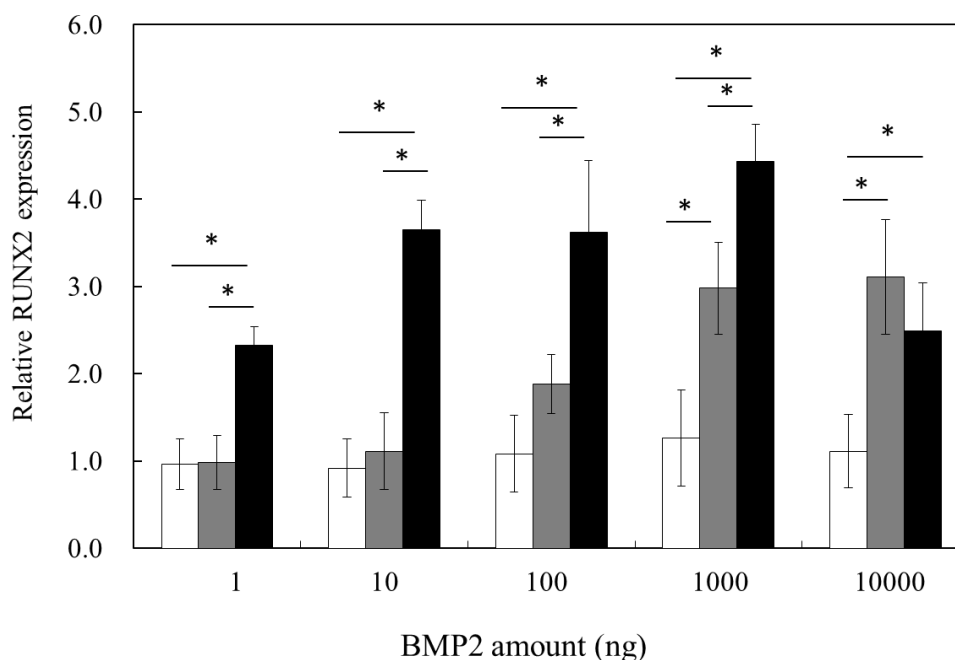


Figure 9. The RUNX2 mRNA expression of MC3T3-E1 cells in aggregates 7 days after incubation with once (□) or 10 times (■) addition of free BMP2 or GM-BMP2 (■). The total amount of BMP2 added were 1, 10, 100, 1000, and 10000 ng. GM were dehydrothermally crosslinked for 18 hr at 140 °C. \*  $p < 0.05$ , significant against the RUNX2 mRNA expression between two groups.

Figures 10 and 11 show the ALP activity and the calcium content of MC3T3-E1 cell in cell aggregates cultured with GM-BMP2. Neither ALP activity nor calcium content was observed for MC3T3-E1 cell aggregates cultured without GM, irrespective of BMP2 presence. After 7 days culture, the ALP activity was significantly high for MC3T3-E1 cell aggregates with GM-BMP2 prepared by crosslinking for 18 hr at 140 °C compared with other experimental groups. After 21 days culture, the significantly higher ALP activity and calcium content were observed for MC3T3-E1

cell aggregates cultured with GM-BMP2 prepared by the crosslinking for 48 and 96 hr.

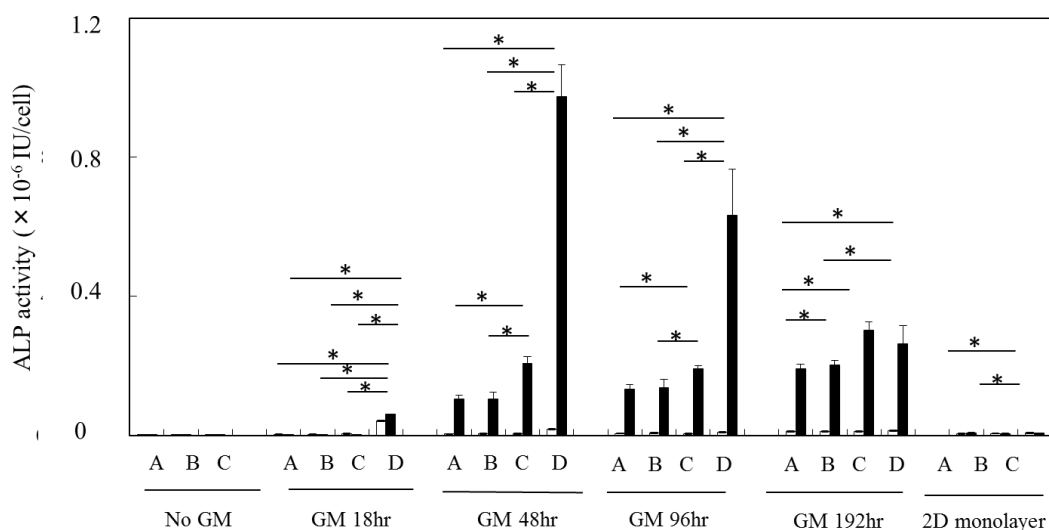


Figure 10. The ALP activity of MC3T3-E1 cells in aggregates without or with GM incorporation 7 (□) and 21 days (■) after incubation in the absence (A) or presence of once (B) or 10 times (C) addition of free BMP2 or GM-BMP2 (D). The total amount of BMP2 added was 10 ng. GM were dehydrothermally crosslinked for 18, 48, 96, and 192 hr at 140 °C. The MC3T3-E1 cells without GM were cultured in each well of flat-bottomed 96-multiwell plates as a 2D monolayer culture in the absence (A) or presence of once (B) or 10 times (C) addition of free BMP2 into the medium. \*  $p < 0.05$ , significant against the ALP activity between two groups.

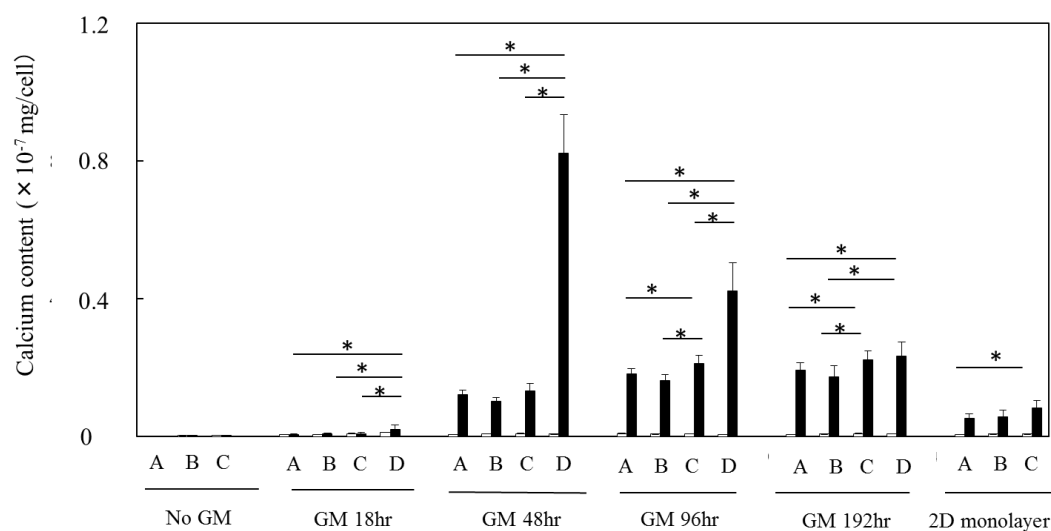


Figure 11. The calcium content of MC3T3-E1 cells in aggregates without or with GM incorporation 7 (□) and 21 days (■) after incubation in the absence (A) or presence of once (B) or 10 times (C) addition of free BMP2 or GM-BMP2 (D). The total amount of BMP2 added was 10 ng. GM were dehydrothermally crosslinked for 18, 48, 96, and 192 hr at 140 °C. The MC3T3-E1 cells without GM were cultured in each well of flat-bottomed 96-multiwell plates as a 2D monolayer culture in the absence (A) or presence of once (B) or 10 times (C) addition of free BMP2 into the medium. \*  $p < 0.05$ , significant against the calcium content between two groups.

### 3.4 Discussion

The previous study demonstrates that the remaining of GM of isoelectric point (IEP) 5.0 gelatin in the MC3T3-E1 cell aggregates at larger amounts and for longer time period allowed MC3T3-E1 cells to enhance their proliferation, ALP activity, and calcium content compared with MC3T3-E1 cell aggregates cultured without GM or in the 2D monolayer system [11]. In this study, GM were prepared from gelatin of IEP 9.0 because better BMP2 released was observed from the gelatin hydrogels of IEP 9.0 than those of IEP 5.0 [47]. The BMP2 was released from the hydrogels in a sustained manner. The release rate increased as the hydrogel degradation rate became faster. The in vitro degradation test revealed that the degradation time of GM in HCl solution increase with an increase in the crosslinking time (Figure 3). It is likely that the crosslinking number of gelatin microspheres increases with an increase in the crosslinking time, resulting in their slower degradation. The size of GM was similar, irrespective of the crosslinking time (Figure 1). It was practically possible for this study to prepare the GM with a similar size but with different degradabilities.

The cell aggregates incorporating gelatin microspheres with different degradabilities were formed in the U-bottomed wells (Figure 4). With the incubation time, the size of MC3T3-E1 cell aggregates cultured with GM tended to reduce. The size change depended on the type of GM incorporated. This can be explained in terms of microspheres degradation. It is highly conceivable that slowly degraded GM remained in the MC3T3-E1 cell aggregates for longer time periods, resulting in no size change or slightly reduced size. The similar phenomenon was observed in the previous research [11]. The order of GM degradation of in MC3T3-E1 cell aggregates was similar to that in HCl solution (data not shown).

To investigate the effect of BMP2 on the osteogenic differentiation of cells, MC3T3-E1 cells cultured in the following three conditions of BMP2 existence. BMP2 was added in the GM incorporated form and one or 10 times sequential addition of free BMP2 combined with GM (Figure 1). The BMP2 distribution in MC3T3-E1 cell aggregates after culture with GM-BMP2 was homogeneous. It is possible that with incubation time, BMP2 would be released from GM-BMP2 homogeneously incorporated in cell aggregates. Consequently, homogeneous BMP2 distribution would be achieved. On the other hand, the BMP2 distribution in MC3T3-E1 cell aggregates cultured with GM plus BMP2 addition was heterogeneous, and a larger amount of BMP2 was localized in the peripheral portion of aggregates (Figure 5). This is due to the poor permeation of BMP2 into cell aggregates. It is reported that there is a limitation of BMP2 diffusion and BMP2 cannot be diffused into a thick tissue over 200–400  $\mu\text{m}$  thickness [48-50]. In this study, it is conceivable that the size of cell aggregates with GM were approximately 400-600  $\mu\text{m}$ , which causes the poor permeation of BMP2 into cell aggregates.

The ratio of L-lactic acid to glucose is a general measure of aerobic metabolism [46]. The lower the L-lactic acid/glucose ratio is, the higher aerobic metabolism is. Irrespective of the presence BMP2, the lactic acid/glucose ratio of cell aggregates incorporating GM-BMP2 of lower degradabilities was significantly lower than that of aggregates without or with GM-BMP2 of higher degradabilities incorporation (Figure 6). The low ratio was as similar as that of monolayer cell cultures, in which oxygen and nutrient are abundantly supplied. Even in the longer time culture, it is conceivable that the larger amount of GM-BMP2 of lower degradabilities remained in the cell aggregates. This longer remaining would increase the oxygen level, resulting in a lower ratio of

L-lactic acid to glucose, namely in an aerobic cell culture condition. In addition, the presence of GM-BMP2 homogeneously distributed in the cell aggregate may increase the oxygen and nutrients permeability into the aggregate. Based on the better conditions of cell culture, we can say with certainty that the number of live cells proliferated in the cell aggregate cultured with GM-BMP2 of lower degradabilities was significantly higher (Figure 7).

For the evaluation of osteogenic differentiation, RUNX2 mRNA expression, ALP activity, and calcium content of cell aggregates were measured [37-42, 51]. BMP2 binds to the BMP receptor (BMPR) on the cell surface, and then the BMPR activates a receptor-specific Smads signal pathway, resulting in an enhanced osteogenesis. In the early osteogenic differentiation, it has demonstrated that BMP-activated Smads and RUNX2 signal are critical for bone formation [38-39, 52]. There have been several reports that the amount of BMP2 released from biomaterials affects the osteogenic differentiation [53-57]. It is possible to promote osteogenic differentiation at larger amount of BMP2 in a nanogram order.

After 7 days culture, higher expression of ALP and RUNX2 for MC3T3-E1 cells in cell aggregates cultured with GM-BMP2 of faster degradation was observed than those of once or 10 times addition of free BMP2 (Figures 8 and 9). This can be explained in terms of BMP2 distribution in cell aggregates and GM degradability. For the cell aggregates incorporating GM-BMP2, BMP2 were homogeneously distributed in cell aggregates, this would result in homogeneous BMP2 activation of osteogenic differentiation even at a small amount of BMP2. The time profile of BMP2 release can be controlled by that of GM degradation [40]. It is likely that the slower degradation of GM-BMP2 achieves the BMP2 release for a longer time period, resulting in a prolonged

existence of BMP2. The prolonged BMP2 existence would act on the cells for a longer time period to enhance the gene expression. On the other hand, for MC3T3-E1 cell aggregates cultured with GM once or 10 times addition of BMP2, BMP2 was localized in the peripheral portion of cell aggregates incorporating GM because of the BMP2 diffusion limitations into aggregates, resulting in poor osteogenic differentiation at a small amount of BMP2. In addition, Higher ALP and RUNX2 expression of MC3T3-E1 cells for MC3T3-E1 cell aggregates cultured with GM 10 times addition of BMP2 were observed than those of once addition of BMP2. It is conceivable that the sequential existence of BMP2 is important for the osteogenic differentiation of MC3T3-E1 cell aggregates. The result for the sequential addition of factors is consistent with that of previous study [58].

After 21 days culture, few GM remained in cell aggregates for the fast-degraded GM-BMP2. As a result, oxygen and nutrients supplies are poor and BMP2 existence for a shorter time period which causes the poor osteogenic differentiation. On the other hand, larger amounts of slow-degraded GM-BMP2 remained in cell aggregates even after 21 days culture. It is possible that the longer remaining of GM gives cells longer supply of oxygen and nutrient and longer-term BMP2 release, resulting in better culture conditions for osteogenic differentiation. However, higher ALP activity and calcium content were observed for MC3T3-E1 cell aggregates incorporating medium-degraded GM-BMP2 than those of slow-degraded GM-BMP2. This can be explained in terms of oxygen tension and the amount of BMP2 released from GM-BMP2. It is recognized that the oxygen tension is one of the important mediators for osteogenic differentiation [59-62]. For MC3T3-E1 cell aggregates incorporating medium- and slow-degraded GM, the oxygen level in the aggregate would remain at the normal level during the long-term

culture. In contrast, for MC3T3-E1 cell aggregates with or without fast-degraded GM incorporatin, the disappearance of GM due to their faster degradation would reduce the oxygen level. The homogeneous distribution of microspheres in cell aggregates would keep the internal oxygen at normal, resulting in the higher viability and osteogenic differentiation of MC3T3-E1 cells. In addition, it is conceivable that higher amounts of BMP2 were released from medium-degraded GM than slow-degraded GM, resulting in better osteogenic differentiation of MC3T3-E1 cell aggregates incorporating medium-degraded GM. Finally, MC3T3-E1 cell aggregates incorporating medium- and slow-degraded GM showed higher osteogenic differentiation compared with monolayer-cultured cells. Better osteogenic differentiation of 3D cell aggregates than the monolayer culture has previously been reported [63]. In this study, we can say with certainty that the 3D cell–cell interactions rendered the cells more sensitive to osteogenic differentiation.

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## **PART II**

### **Design of Gelatin hydrogel or Matrigel Microspheres to Enhance Cell Viability and Functions of Epithelial Cell Aggregates**



## **Chapter 4**

### **Preparation of epithelial cell aggregates incorporating matrigel microspheres to enhance proliferation and differentiation of epithelial cells**

#### **4.1 Introduction**

Recently, cell researches have become more and more popular to clarify the molecular mechanisms of cell proliferation and differentiation. The epithelium is the first emerging tissue during ontogenesis, and epithelial cells play fundamental roles in embryo morphogenesis and organ development [1–5]. Epithelial cells have segregated apical and basolateral plasma membrane domains with asymmetric compositions of nutrient and fluid transporters which are required to carry out crucial vectorial transport functions and cytoplasmic polarity to generate different cell progenies for tissue morphogenesis [6–7]. However, there have been some problems by the culture of epithelial cells. In two-dimensional (2D) cell culture systems on a plastic plate, epithelial cells quickly lose their functions, and do not always proliferate as well as other types of cells. Because the local environment of epithelial cells is different from that of mesenchymal cells in living tissues [8]. As one tried to tackle this problems, epithelial cells are cultured with the feeder layer of fibroblasts for their proliferation, but their functions are biologically insufficient because of the lack of basement membrane components [9–11]. In three-dimensional (3D) cell culture systems, epithelial cells are often cultured with 3D basement membrane component-rich gels [12–13]. Cell aggregates are formed with a central lumen and polarized structures, but cells are not proliferated well, while cells in center of aggregates die by apoptosis [14–18]. We demonstrate that mouse preosteoblast MC3T3-E1 cells were cultured with gelatin

hydrogel microspheres (GM) to form the MC3T3-E1 cell aggregates homogeneously incorporating GM for an enhanced cell proliferation and osteogenic differentiation [19]. The GM incorporation enabled cells to rescue the lack of oxygen in cell aggregates.

In the physiological condition, most cells are present in a 3D structure in which the cell–cell and cell-extracellular matrix interactions are naturally to allow cells to survive and biologically function [20]. This 3D structure of cells is important and essential to promote their functions. For example, embryonic stem cells generally aggregate to form an embryoid body, and consequently initiate their differentiation into different cell lineages [21]. The aggregation of liver cells to form a spheroid is necessary to enhance their metabolic activity [22]. Cell aggregates produce extracellular matrix proteins more efficiently than single cells [23]. Considering the cell structure of body tissues, such as liver and bone, cell aggregates biologically function as the minimum unit [24].

The objective of this study is to prepare a new 3D aggregates culture system of epithelial cells for an enhanced cell proliferation and differentiation. In this study, matrigel microspheres (MM) and matrigel-coated GM were prepared. Mouse mammary epithelial EpH4 cells were cultured with the microspheres to form cell aggregates homogeneously incorporating microspheres to evaluate the proliferation and differentiation in terms of the expression of differentiation markers. We examine the effect of MM, GM, and matrigel-coated GM on the cell behavior.

## 4.2 Materials and methods

### 4.2.1 Preparation of matrigel microspheres

Matrigel microspheres (MM) were prepared by a coacervation method [25]. Briefly, 1.0 ml of 10 vol% aqueous Becton, Dickinson and Company (BD) Matrigel™ Basement Membrane Matrix (BD Biosciences, Inc., Franklin Lakes, America) solution was prepared at 4 °C. Then, 4 ml of 2-butanol (Nacalai Tesque, Inc. Kyoto, Japan) was added to the matrigel solution at 4 °C. The resulting microspheres were gelationed for 1 hr at 37 °C. Then, 2-butanol was removed by evaporation, and followed by centrifuged for 5 min at 14,000 rpm at 4 °C to obtain MM. The MM were stored at -30 °C until to use.

### 4.2.2 Preparation of gelatin hydrogel microspheres

Gelatin hydrogel microspheres (GM) were prepared by the chemical crosslinking of gelatin in a water-in-oil emulsion state according to the method previously reported [26]. Briefly, an aqueous solution (20 ml) of 10 wt% gelatin (isoelectric point 5.0, weight-averaged molecular weight 1,00,000, Nitta Gelatin Inc., Osaka, Japan) was preheated at 40 °C, and then added dropwise into 600 ml of olive oil (Wako Ltd, Osaka, Japan) at 40 °C, followed by stirring at 400 rpm for 10 min to prepare a water-in-oil emulsion. The emulsion temperature was decreased to 4 °C for the natural gelation of gelatin solution to obtain non-crosslinked hydrogel microspheres. The resulting microspheres were washed three times with cold acetone in combination with centrifugation (5000 rpm, 4 °C, 5 min) to completely exclude the residual oil. Then, they were fractionated by size using sieves with apertures of 20 µm (Iida Seisakusyo

Ltd, Osaka, Japan) and air dried at 4 °C. The non-crosslinked and dried gelatin hydrogel microspheres (200 mg) were treated in a vacuum oven at 140 °C and 0.1 Torr for the dehydrothermal crosslinking of gelatin for 24 hr according to the method previously reported [19]. The pictures of gelatin hydrogel microspheres in the water swollen state were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan). The size of 100 microspheres for each sample was measured using the computer program Image J (NIH Inc., Bethesda, USA) to calculate the average size.

#### ***4.2.3 Preparation of matrigel-coated gelatin hydrogel microspheres***

The matrigel solution (20 µl) was dropped onto 2 mg of freeze-dried gelatin hydrogel microspheres (GM), followed by leaving at 4 °C for overnight to allow matrigel to absorb onto the microspheres. The matrigel solution was completely absorbed into the GM because the solution volume was much less than that theoretically required for the equilibrated swelling of microspheres. To evaluate the matrigel existence on the surface of matrigel-coated GM were incubated with an anti-laminin antibody (Abcam Inc., Cambridge, UK) for 60 min at 25 °C and subsequently Alexa Fluor® 488 Donkey anti-rabbit (Thermo Fisher Inc., Massachusetts, America) for 30 min at 25 °C, followed by fluorescent viewing with confocal laser scanning microscope (FV1000D, Olympus Ltd, Tokyo, Japan)

#### ***4.2.4 Cell culture experiments***

EpH4 cells of a mouse mammary epithelial cell line were transfected and clones selected as previously described [27]. EpH4 were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Thermo Inc., Waltham, USA)

supplemented with 2 vol% fetal calf serum (FCS) (Thermo Inc., Waltham, USA), gentamicin, 3 µg/ml prolactin (Sigma-Aldrich Inc., St. Louis, America), 1 µg/ml hydrocortisone (Sigma-Aldrich Inc., St. Louis, America) and 5 µg/ml insulin (Sigma-Aldrich Inc., St. Louis, America) (standard medium) and cultured at 37 °C in a 95% air-5% CO<sub>2</sub> atmosphere. The culture medium was changed every 2 days and confluent cells were subcultured through trypsinisation. In the experiment of phosphatidylinositol 3-kinase (PI3K, Sigma-Aldrich Inc., St. Louis, America) inhibition experiment of EpH4 cells, the standard medium containing a PI3K inhibitor LY294002 (50 µM, Abcam Inc., Cambridge, British) was used and the cells were cultured for 72 hr.

#### ***4.2.5 Preparation of cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres***

Poly(vinyl alcohol) (PVA, 1800 degree of polymerization and 88 mol% saponification) kindly supplied from Unichika (Tokyo, Japan) was dissolved in 1X Dulbecco's phosphate buffered saline (PBS, Nissui Ltd, Tokyo, Japan) to give a concentration of 1 wt%. The PVA solution (100 µl/well) was added to each well of 96-multiwell culture plate with either flat- or round-bottomed (U-bottomed) wells and incubated at 37 °C for 15 min. Then, the solution was removed by aspiration and the wells were washed twice with PBS (100 µl/well). MM, GM or matrigel-coated GM and EpH4 cells were separately suspended in the standard medium. The microspheres-free standard medium or the suspensions of MM, GM, and matrigel-coated GM (0,  $2 \times 10^3$ ,  $2 \times 10^4$ ,  $2 \times 10^5$  microspheres/ml) (50 µl/well) were added to the PVA-coated wells, followed by the addition of EpH4 cells suspension ( $2 \times 10^5$  cells/ml, 50 µl/well). For the

conventional 3D epithelial cells culture, 10  $\mu$ l of matrigel solution was placed into the flat-bottomed wells of 96-well flat-bottomed culture plates at 4 °C, and then the culture plates were incubated at 37°C for 15 min for matrigel polymerization. Next, EpH4 cells suspension ( $1 \times 10^5$  cells/ml, 100  $\mu$ l/well) containing 2 vol% matrigel was added in the matrigel-treated wells. The pictures of cells 3D cultured with were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan).

#### ***4.2.6 Evaluation of cells viability in cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres***

Live/dead assays were conducted using Live/Dead<sup>®</sup> Viability/Cytotoxicity assay (Invitrogen inc., , Carlsbad, UK) according to the manufacture's protocol. After 7 days culture, cell aggregates were rinsed with PBS, and then incubated with a solution containing 2  $\mu$ M calcein AM and 4  $\mu$ M EthD-1 in PBS for 30 min at 37 °C in the dark, followed by fluorescent viewing with the confocal laser scanning microscope.

#### ***4.2.7 Measurement of live cells number in cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres***

The number of live cells in EpH4 cell aggregates with MM, GM or matrigel-coated GM incorporation was determined by counting the number of cell nuclei after the crystal violet staining [28]. Briefly, a mixed solution of 0.2 M citric acid and 0.2 wt% crystal violet was added (100  $\mu$ l/well) to each well of well plate 7 days after EpH4 cell culture with MM, GM, and matrigel-coated GM. After crystal violet staining the cells were lysed in 0.1 wt% Triton X-100 in PBS at 37 °C overnight to extract the nuclei from the cells. After pipetting, the nuclei collected were viewed under a microscope

(CKX31-11PHP, Olympus Ltd, Tokyo, Japan) and counted in a hemocytometer (OneCell Inc., Hiroshima, Japan). The nuclei of live cells are generally round, while dead cell nuclei are irregularly shaped. Base on the nucleus shape, live cells can be distinguished from dead cells to assess the number of live cells.

#### ***4.2.8 Measurement of L-lactic acid/glucose ratio of cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres***

EpH4 cells were similarly cultured with MM, GM, or matrigel-coated GM to form the cell aggregates. The amount of glucose consumed by EpH4 cell aggregates was determined by measuring the change in glucose concentration in the culture medium using a Glutest Neo Super test kit (Sanwa kagaku kenkyusyo Ltd, Kyoto, Japan) 7 days after incubation. Similarly, the amount of L-lactic acid produced by EpH4 cell aggregates was determined with an E-kit (R-Biopharm AG Ltd, Germany) 7 days after incubation. The number of live cells was determined by the crystal violet staining as described above and used to normalize the amount of glucose consumption and L-lactic acid produced by the number of live cells. The L-lactic acid/glucose ratio was calculated as a measure of the aerobic metabolism of cells [29].

#### ***4.2.9 Immunostaining of cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres***

EpH4 cells were similarly cultured with MM, GM, or matrigel-coated GM to form the cell aggregates. After incubation for 7 days, the cell aggregates were fixed with 4 vol% paraformaldehyde at 4 °C for 1 hr and embedded in optimal cutting temperature

compound (Sakura Finetek Japan Ltd, Tokyo, Japan) and frozen in liquid nitrogen. The frozen samples were sectioned using a cryotome (CM3050S, Leica Microsystems, Wetzlar, Germany) and incubated at 4°C overnight with the following primary antibodies:  $\beta$ -casein (Santacruz Inc., America, 1:50) or Laminin (Abcam Inc., Cambridge, UK, 1:100). Then, secondary antibodies coupled to Alexa 488 (Molecular Probes, Invitrogen Inc. Carlsbad, America, 1:700) were incubated at 25 °C for 30 min for fluorescent viewing. Next, TO-PRO-3 (Molecular Probes, Eu-gene, USA) was added to incubate at 25 °C for 10 min for the nuclear staining of cells. The sections of 10  $\mu$ m thickness were viewed in a confocal laser scanning microscope (FV1000D, Olympus Ltd, Tokyo, Japan).

#### ***4.2.10 Measurement of $\beta$ -casein and E-cadherin expression in cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres***

The messenger ribonucleic acid (mRNA) expression of  $\beta$ -casein and E-cadherin was evaluated by real-time polymerase chain reaction (PCR). Briefly, EpH4 cells were cultured with MM, GM, or matrigel-coated GM to form the cell aggregates. After incubation for 7 days, RNA was extracted and cleaned up using the Qiagen RNeasy® Plus Mini kit (Qiagen Ltd, Hilden, Germany) according to the manufacturer's instructions. The total RNA sample was reverse-transcribed to cDNA using SuperScript VILO™ (Invitrogen, Carlsbad, CA). The resulting cDNA was then subjected to real-time PCR by using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems inc., Carlsbad, CA) to determine the gene expression. The PCR reaction was carried out with specific primers in the presence of Power SYBR Green (Applied

Biosystems Inc., Carlsbad, CA) as described in the manufacturer's instruction. The level of gene expression was normalized by that of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, and calculated as the ratio to that of EpH4 cell culture without microspheres of a control group. Experiments were performed for 4 specimens independently for each sample. The primer sequences for GAPDH and  $\beta$ -casein were as follows, GAPDH:

5-TCCACAAGGACAGAGTCAGATTAC-3, 5-TGGCTCAGATAGGAGGGGTA-3;

$\beta$ -casein: 5-GGTGAATCTCATGGGACAGC-3,

5-TGACTGGATGGTGGAGTGAA-3; E-cadherin:

5-CAGAATGACAACAGGCCAGA, 5-TTCATCACGGAGGTTGGTG.

#### **4.2.11 Statistical analysis**

All the statistical data are expressed as the mean $\pm$ standard error of the mean (SEM). The data were analyzed by t-test to determine the statistical significance of differences between two mean values, which was accepted at  $<0.05$ .

### 4.3 Results

#### 4.3.1 Characterization of matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres

Figure 1 shows the microscopic pictures of MM and GM dispersed in water. The microspheres were of spherical shape and had a smooth surface. The size of the matrigel and gelatin microspheres in the swollen condition was  $19.0 \pm 6.5$  and  $16.0 \pm 5.6$   $\mu\text{m}$ , respectively.

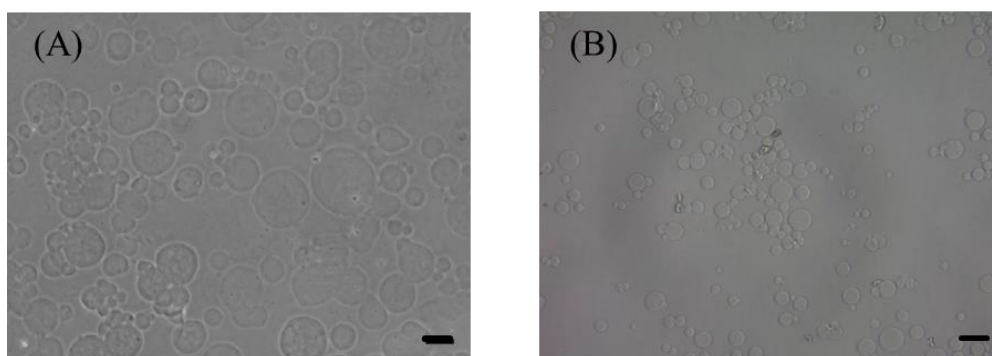


Figure 1. Light microscopic pictures of MM and GM dispersed in water.

The GM were dehydrothermally crosslinked for 18 hr at 140 °C. Scale bar.

20  $\mu\text{m}$ .

Figure 2 shows the microscopic picture of matrigel-coated GM dispersed in water. Matrigel was localized on the surface of GM. The size of matrigel-coated GM was  $17.0 \pm 5.8$   $\mu\text{m}$ . The size and aggregate formation of GM were not changed by the matrigel coating.

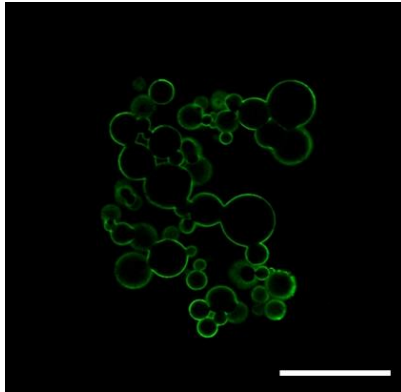


Figure 2. A confocal microscopic picture of matrigel-coated GM dispersed in water. The GM were dehydrothermally crosslinked for 18 hr at 140°C. The laminin of matrigel was immunostained in green. Scale bar. 100  $\mu$  m.

#### ***4.3.2 Formation of cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres***

Figure 3 shows the microscopic pictures of EpH4 cell aggregates 7 days after incubation without or with MM, GM, and matrigel-coated GM in the U-bottomed wells of culture plate, and EpH4 cells cultured by other methods. EpH4 cell aggregates were not formed when the number of microspheres added initially was  $1 \times 10^4$ /well. EpH4 cell aggregates were formed for cultured without or with the microspheres added number of  $1 \times 10^2$  and  $1 \times 10^3$ /well and the conventional 3D method. The shape of EpH4 cell aggregates was of spherical when the number of MM added initially was  $1 \times 10^3$ /well or for the conventional 3D method. However, the shape of EpH4 cell aggregates was not of spherical, but oval shape for cell aggregates cultured without or with  $1 \times 10^2$  of MM, GM, and matrigel coated GM.

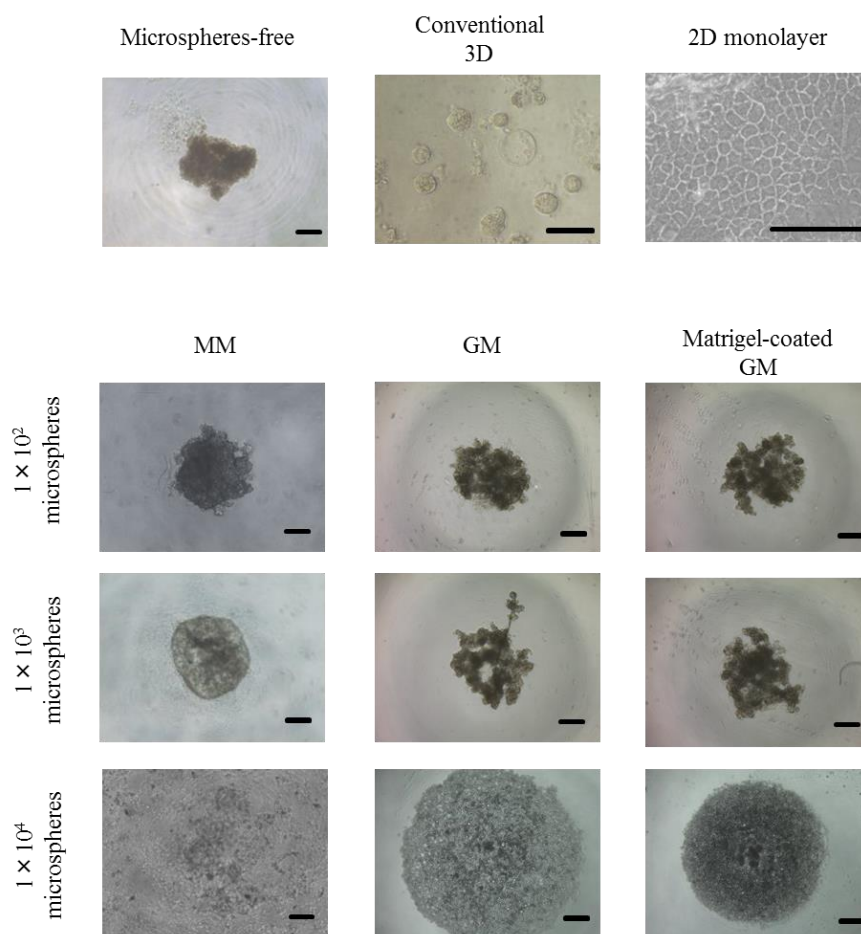


Figure 3. Light microscopic pictures of EpH4 cell aggregates 7 days after incubation of EpH4 cells with MM, GM, and matrigel-coated GM or without microspheres. EpH4 cells were cultured as by the conventional 3D and 2D monolayer culture methods. The number of EpH4 cells added initially was  $1 \times 10^4$ /well while that of microspheres was  $1 \times 10^2$ ,  $1 \times 10^3$  or  $1 \times 10^4$  /well. Scale bar. 100  $\mu\text{m}$ .

#### 4.3.3 Cell viability of cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres

Figure 4 shows the fluorescent pictures of EpH4 cell aggregates 7 days after

incubation without or with MM, GM, and matrigel-coated GM incorporatin. Irrespective of the culture method, EpH4 cells in cell aggregates were alive.

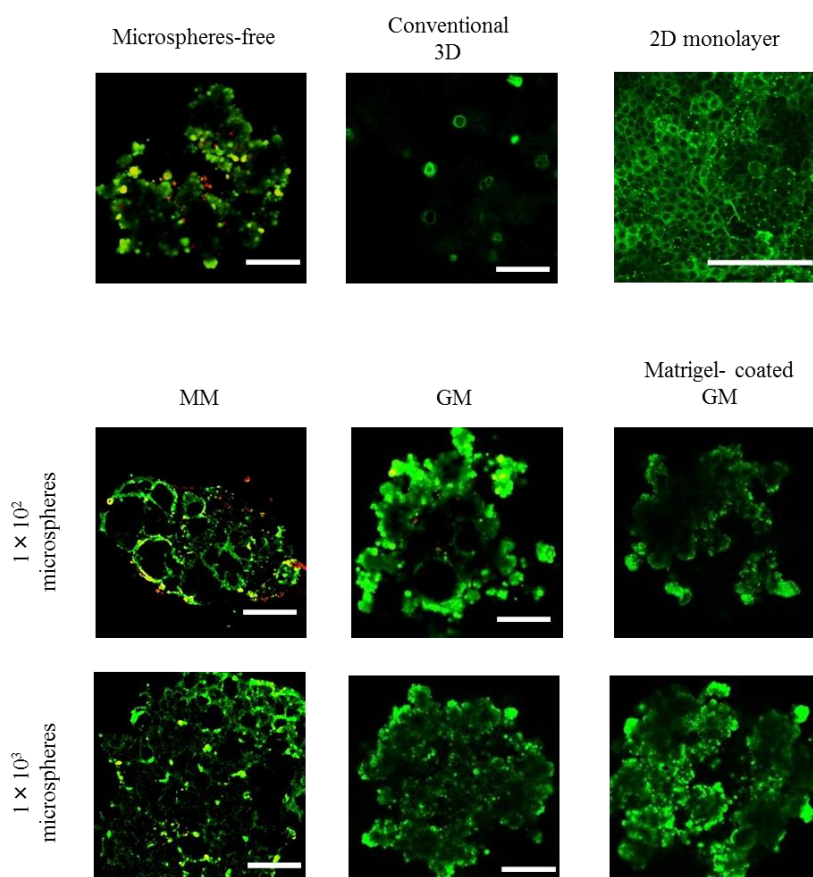


Figure 4. Live/dead assay of EpH4 cell aggregates 7 days after incubation of EpH4 cells with MM, GM, and matrigel-coated GM or without microspheres. EpH4 cells were cultured by the conventional 3D and 2D monolayer methods. The number of EpH4 cells added initially was  $1 \times 10^4$ /well while that of microspheres was  $1 \times 10^2$  or  $1 \times 10^3$ /well. Scale bar. 100  $\mu\text{m}$ .

#### 4.3.4 Live cells number of cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres

Figure 5 shows the number of live EpH4 cells 7 days after incubation without or with MM, GM, and matrigel-coated GM incorporation, and cultured by the conventional 3D and 2D monolayer culture methods. The number of live EpH4 cells of cell aggregates incorporating  $1 \times 10^3$  of MM, GM, and matrigel-coated GM was significantly high compared that of cell aggregates without microspheres incorporation and the conventional 3D method. However, the number was lower than that of 2D culture method.

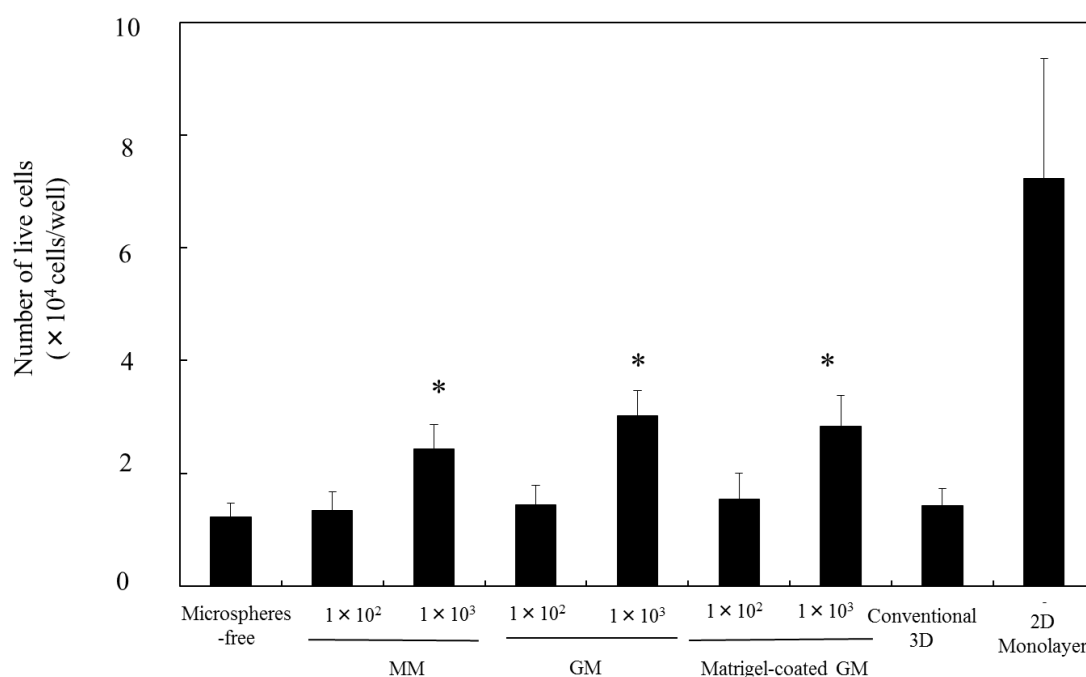


Figure 5. The number of live EpH4 cells 7 days after incubation of EpH4 cells with MM, GM, and matrigel-coated GM or without microspheres. EpH4 cells were cultured by the conventional 3D and 2D monolayer methods. The number of EpH4 cells added initially was  $1 \times 10^4$ /well while

that of microspheres was  $1 \times 10^2$  or  $1 \times 10^3$ /well. \*,  $p < 0.05$ ; significant against the number of live EpH4 cells in cell aggregates cultured without microspheres.

#### 4.3.5 L-lactic acid/glucose ratio of cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres

Figure 6 shows the L-lactic acid/glucose ratio of EpH4 cells in EpH4 cell aggregates 7 days after incubation with or without MM, GM, and matrigel-coated GM. The higher L-lactic acid/glucose ratio was observed for EpH4 cells in cell aggregates without microspheres, although there was no significant difference in the L-lactic acid/glucose ratio among the experiment groups.

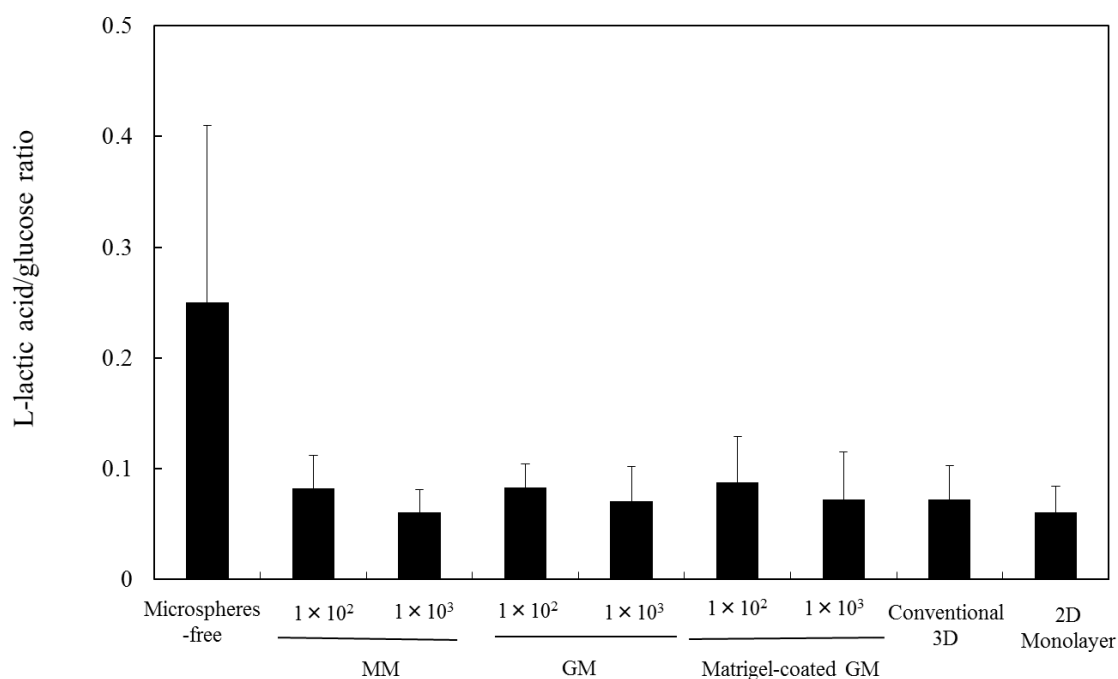


Figure 6. The L-lactic acid/glucose ratio of live EpH4 cells 7 days after incubation of EpH4 cells with MM, GM, and matrigel-coated GM or

without microspheres. EpH4 cells were cultured by the conventional 3D and 2D monolayer methods. The number of EpH4 cells added initially was  $1 \times 10^4$ /well while that of microspheres was  $1 \times 10^2$  or  $1 \times 10^3$ /well.

#### ***4.3.6 Laminin and $\beta$ -casein expression of cell aggregates incorporating matrigel microspheres, gelatin microspheres, and matrigel-coated gelatin microspheres***

Figures 7 and 8 show the fluorescent microscopic pictures of laminin and  $\beta$ -casein of cell aggregates 7 days after incubation without or with MM, GM, and matrigel-coated GM incorporation, and cells cultured by the conventional 3D and 2D monolayer culture methods. The higher expression of  $\beta$ -casein was observed for EpH4 cell aggregates incorporating  $1 \times 10^3$  of MM compared with other groups. For the conventional 3D culture method, the expression of  $\beta$ -casein was observed, but the extent of  $\beta$ -casein expression was lower than that of EpH4 cell aggregates incorporating  $1 \times 10^3$  of MM. The poor expression of  $\beta$ -casein was observed for EpH4 cell aggregates without or with GM and matrigel-coated GM incorporation. The higher expression of laminin was observed for EpH4 cell aggregates incorporating  $1 \times 10^3$  of MM, and the conventional 3D culture method. The expression of laminin was higher for EpH4 cell aggregates incorporating  $1 \times 10^3$  of MM than those of  $1 \times 10^2$  of MM. The expression of laminin was poor for EpH4 cell aggregates incorporating matrigel-coated GM.

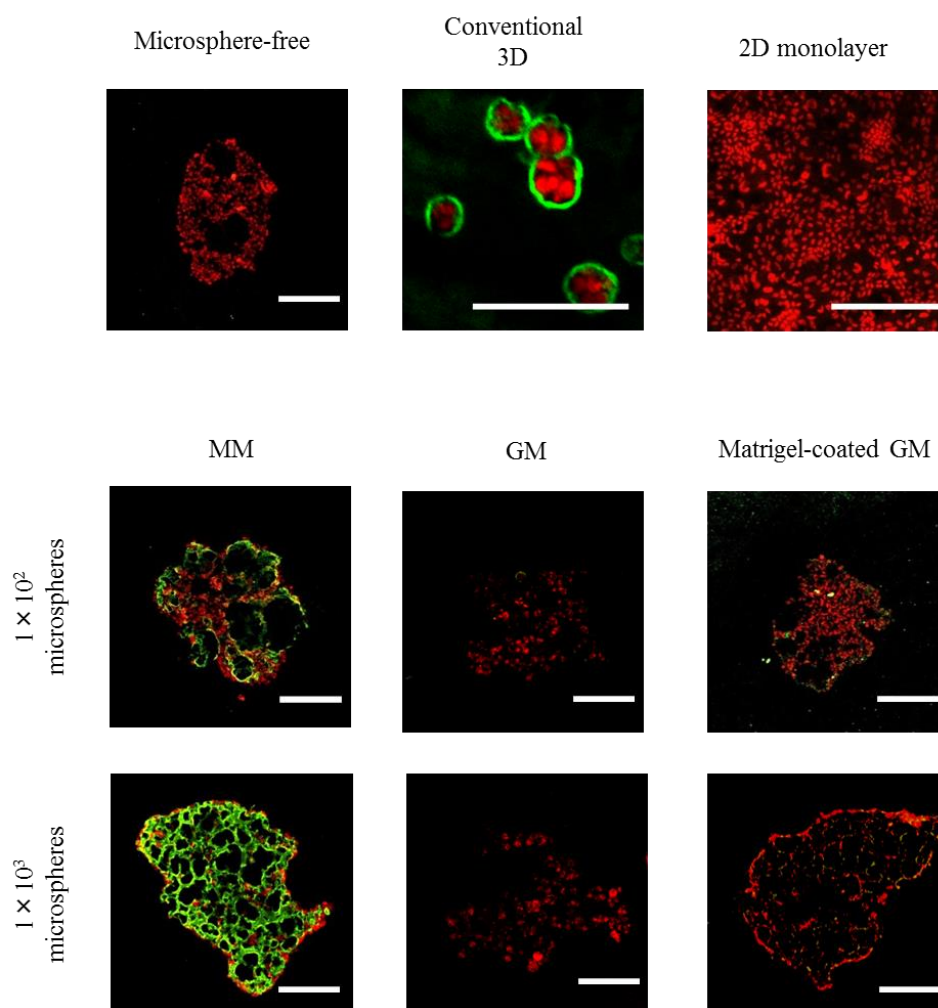


Figure 7. Confocal microscopic pictures of laminin expression of EpH4 cells 7 days after incubation of EpH4 cells with MM, GM, and matrigel-coated GM or without microspheres. EpH4 cells were cultured by the conventional 3D and 2D monolayer methods. The number of EpH4 cells added initially was  $1 \times 10^4$ /well while that of microspheres was  $1 \times 10^2$  or  $1 \times 10^3$ /well. Scale bar. 100  $\mu$ m.

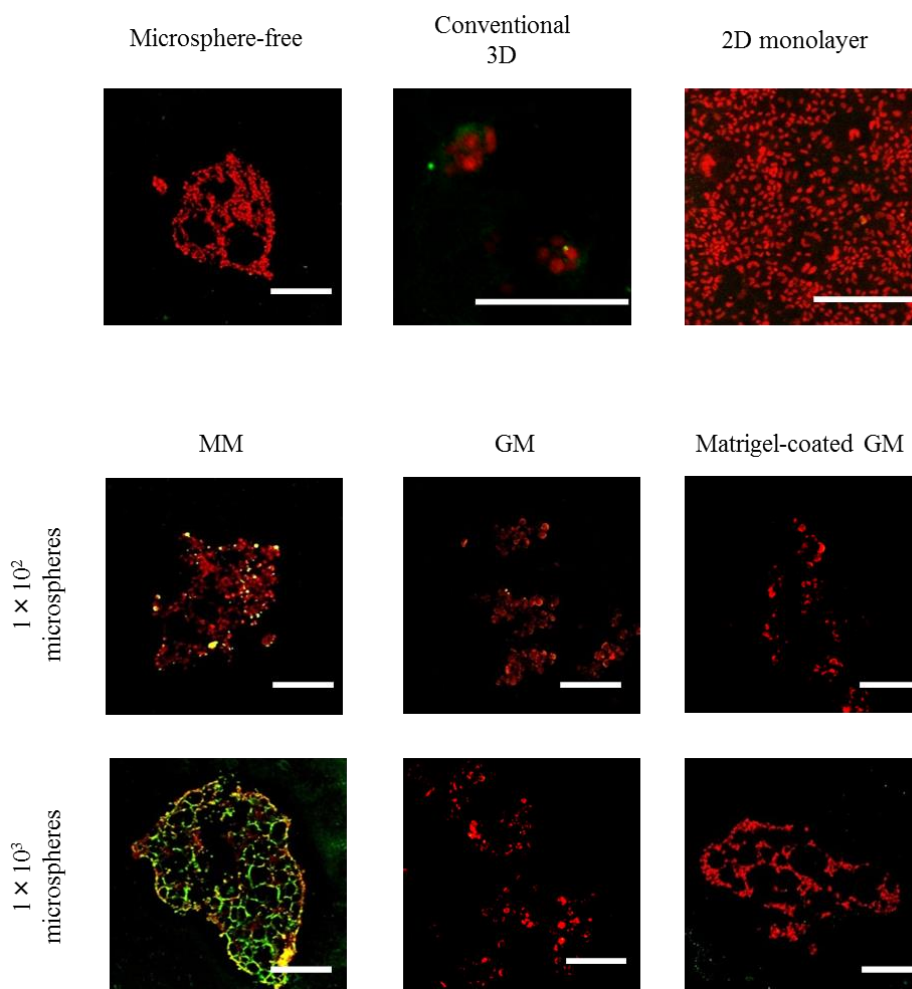


Figure 8. Confocal microscopic pictures of  $\beta$ -casein expression of EpH4 cells 7 days after incubation of EpH4 cells with MM, GM, and matrigel-coated GM or without microspheres. EpH4 cells were cultured by the conventional 3D and 2D monolayer methods. The number of EpH4 cells added initially was  $1 \times 10^4$ /well while that of microspheres was  $1 \times 10^2$  or  $1 \times 10^3$ /well. Scale bar. 100  $\mu$ m.

#### ***4.3.7 $\beta$ -casein and E-cadherin expression of cell aggregates incorporating MM, GM, and matrigel-coated GM***

Figure 9 shows the  $\beta$ -casein expression of EpH4 cell aggregates 4, 7, and 14 days after incubation without or with MM, GM, and matrigel-coated GM and EpH4 cells cultured by the conventional 3D and 2D monolayer culture methods. The  $\beta$ -casein expression of cells cultured by the 3D culture method was higher than that of microspheres-free and 2D monolayer culture methods. The higher Expression of  $\beta$ -casein was observed for cell aggregates incorporating  $1 \times 10^3$  of MM than that of 3D culture methods.

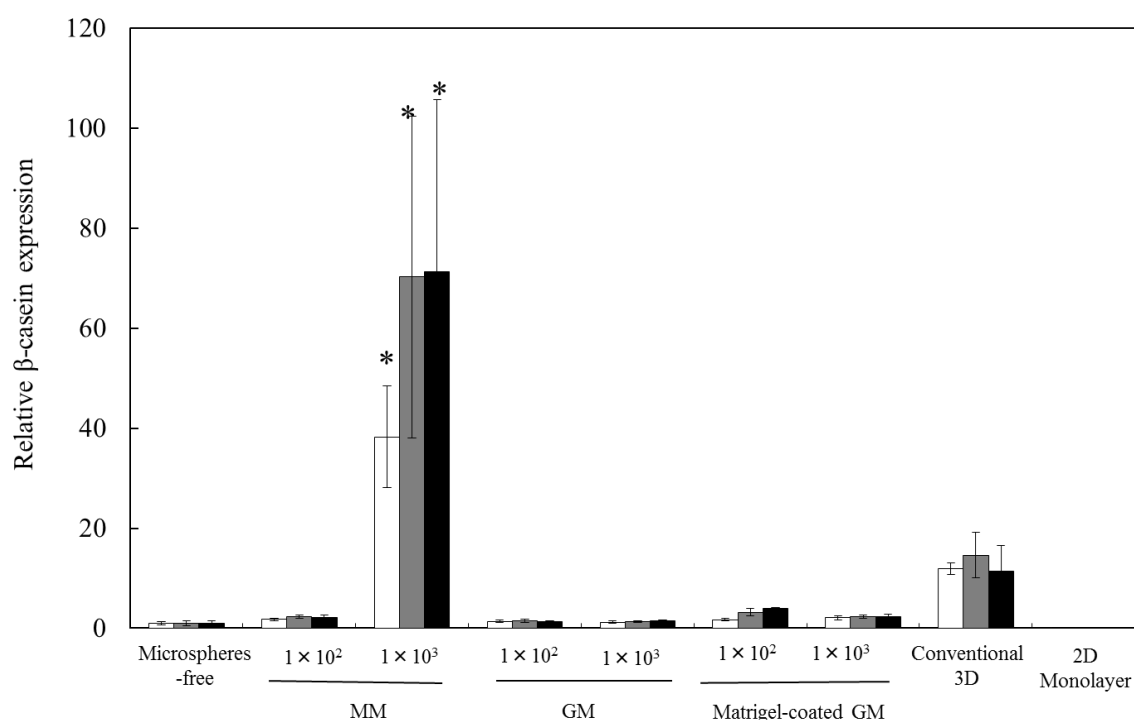


Figure 9. The  $\beta$ -casein expression of EpH4 cells 4 ( $\square$ ), 7 ( $\blacksquare$ ), and 14 days ( $\blacksquare$ ) after incubation of EpH4 cells with MM, GM, and matrigel-coated GM or without microspheres. EpH4 cells were cultured by the conventional 3D and 2D monolayer methods. The number of EpH4 cells added initially was  $1 \times 10^4$ /well while that of microspheres was  $1 \times 10^2$  or

$1 \times 10^3$ /well. \*,  $p < 0.05$ ; significant against the other groups at corresponding time points.

Figures 10 and 11 show the  $\beta$ -casein and E-cadherin expressions of EpH4 cell aggregates 7 days after incubation without or with MM, GM, and matrigel-coated GM incorporation and EpH4 cells cultured by the conventional 3D and 2D monolayer culture methods in the presence or absence of LY294002. For the culture without LY294002 addition, no difference in the expression level of E-cadherin was observed in any experimental conditions. Irrespective of the culture method, the expression of  $\beta$ -casein and E-cadherin significantly was inhibited by the LY294002 addition.

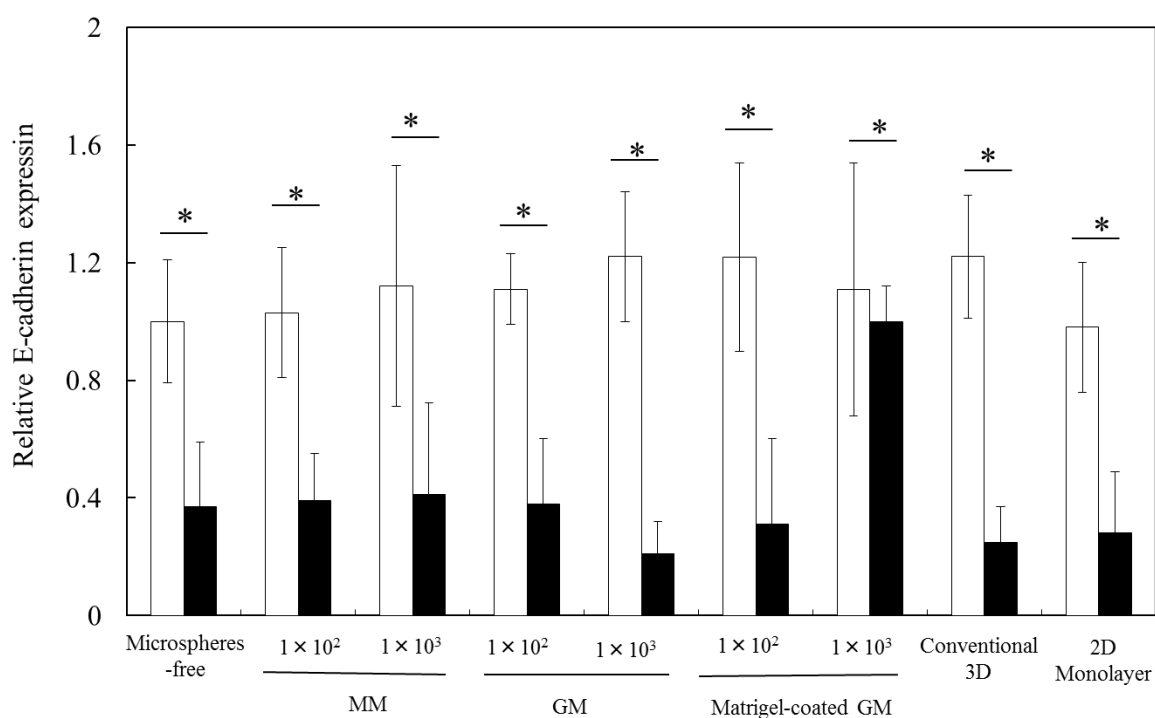


Figure 10. The E-cadherin expression of EpH4 cells 7 days after incubation of EpH4 cells with MM, GM, and matrigel-coated GM or

without microspheres and EpH4 cells were cultured by the conventional 3D and 2D monolayer methods in the absence ( $\square$ ) or presence of PI3K inhibitor LY294002 ( $\blacksquare$ ). The number of EpH4 cells added initially was  $1 \times 10^4$ /well while that of microspheres was  $1 \times 10^2$  or  $1 \times 10^3$ /well. \*,  $p < 0.05$ ; significant against between two groups.

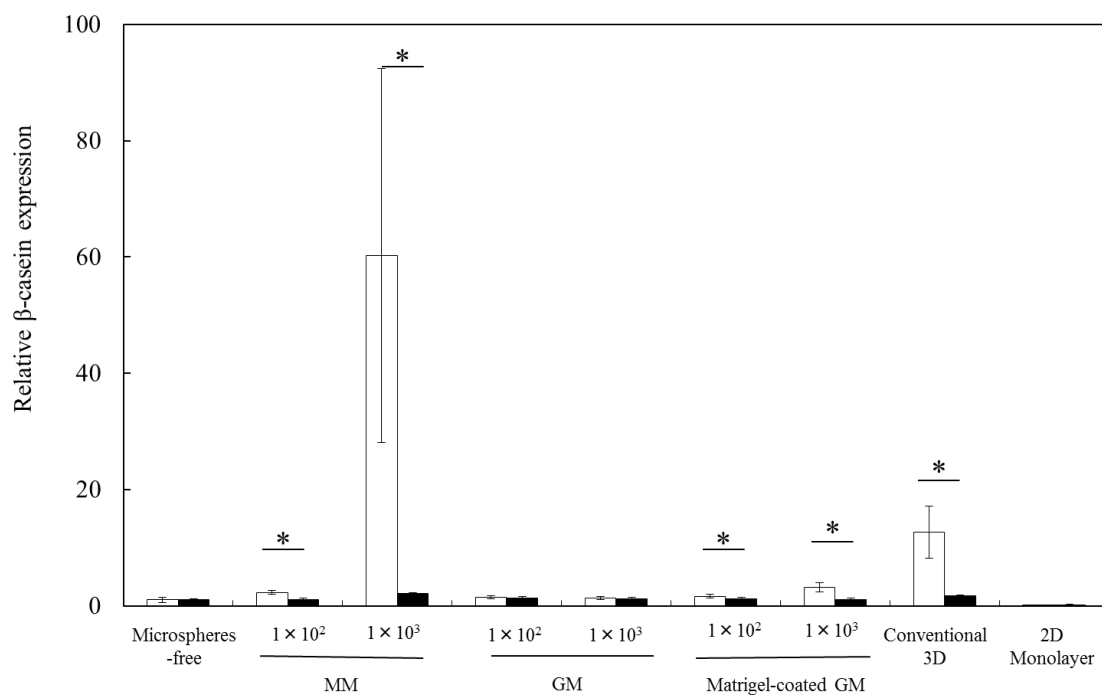


Figure 11. The  $\beta$ -casein expression of EpH4 cells 7 days after incubation of EpH4 cells with MM, GM, and matrigel-coated GM or without microspheres and EpH4 cells were cultured by the conventional 3D and 2D monolayer methods in the absence ( $\square$ ) or presence of PI3K inhibitor LY294002 ( $\blacksquare$ ). The number of EpH4 cells added initially was  $1 \times 10^4$ /well while that of microspheres was  $1 \times 10^2$  or  $1 \times 10^3$ /well. \*,  $p < 0.05$ ; significant against between two groups.

#### 4.4 Discussion

The size of microspheres affects the behavior and functions of cell aggregates. In this study, thus, microspheres with a similar diameter were prepared to exclude the effect of microspheres size (Figure 1). The matrigel coating did not change the size of GM. The fluorescent observation indicated that the laminin of a matrigel component was localized on the surface of GM (Figure 2). Taken together, it is possible that three types of MM, GM, and matrigel-coated GM are good microspheres samples to evaluate the influence of microsphere nature on the biological behavior of cell aggregates.

EpH4 cell aggregates incorporating GM and matrigel-coated GM were formed, but were not of spherical and oval shape. On the other hand, EpH4 cell aggregates incorporating  $1 \times 10^3$  of MM and prepared by the conventional 3D culture method were of spherical shape (Figure 3). The reason why the shape of EpH4 cell aggregates was different is unclear at present. It is conceivable that the oval shape of EpH4 cell aggregates may be caused by the lack of matrigels and a strong cell-cell contact. Another study reported that the matrigel/integrin signaling transduction and strong cell-cell contact were important for morphogenetic changes and the formation of spherical structures [30-32].

The number of live EpH4 cells in cell aggregates incorporating  $1 \times 10^3$  of MM, GM, and matrigel-coated GM was significantly high compared that of the conventional 3D culture method (Figure 5). However, the number of live EpH4 cells was much higher for the 2D culture method than other experimental groups. This may be due to the difference of surface area between microspheres and culture plates as the scaffold of cell proliferation. The surface area of 2D culture plates was much higher than that of  $1 \times 10^3$  of microspheres, resulting in higher proliferation of cells in 2D culture plates. In

addition, the cell proliferation was observed for EpH4 cell aggregates incorporating larger number of microspheres. This results obtained in this study is similar to the results previously reported [33]. The microspheres would act on the scaffold of cell proliferation. The proliferation was not influenced by the type of microspheres incorporated (Figure 5).

The ratio of L-lactic acid to glucose is a general measure of aerobic metabolism [29]. The lower the ratio is, the higher the aerobic metabolism of cell aggregates is. In the previous study, lower L-lactic acid/glucose ratio was observed for cell aggregates with GM than that of cell aggregates without GM. However, in this study, the L-lactic acid/glucose ratio of cells aggregates incorporating MM, GM, and matrigel-coated GM was similar to that of microspheres-free cell aggregates (Figure 7). This can be explained by the shape of cell aggregates. Mesenchymal cell aggregates without GM were of spherical shape and the thickness of cell aggregates over 150 $\mu$ m, which results in lack of the oxygen and nutrients supply into aggregates [19,33]. However, in this study, the epithelial cell aggregates without GM were of not spherical, but oval shape, and the thickness of cell aggregates was smaller than 100 $\mu$ m.

Prolactin is an important hormone to stimulate the milk synthesis of mammary epithelial cells. The signals through the Jak2–Stat5 pathway to induce the expression of a milk protein, such as  $\beta$ -casein, which is often used as a marker for mammary differentiation [32-34]. In addition, cells adhesion to basement membrane proteins is required for the expression of milk protein genes [37-39]. In the evaluation study of laminin,  $\beta$ -casein, and E-cadherin expression of EpH4 cells in cell aggregates or cultured by other methods, the higher expression of laminin and  $\beta$ -casein was observed for EpH4 aggregates incorporating  $1 \times 10^3$  of MM than that of aggregates incorporating

$1 \times 10^2$  of MM and matrigel-coated GM. This can be explained by the amount of laminin in EpH4 cell aggregates incorporating microspheres. Another study reported that the larger amount of laminin enhanced the expression of  $\beta$ -casein of epithelial cells [38,40]. Laminin is one of the major matrigels components. The amount of laminin for  $1 \times 10^3$  of MM was larger than that  $1 \times 10^2$  of MM and matrigel-coated GM (data not shown). After 7 days culture of cell aggregates, no laminin expression of EpH4 cell aggregates was observed for the matrigel-coated GM. It is likely that GM were degraded by enzymes secreted from EpH4 cells, leading to a decrease in the matrigel amount on GM. In addition, it is conceivable that the stiffness of MM is different from that of matrigel-coated GM, resulting in different cell responses. A small laminin amount and different stiffness of matrigel-coated GM incorporated into EpH4 cell aggregates would lead to poor  $\beta$ -casein expression compared with that of MM. The higher  $\beta$ -casein expression was observed for EpH4 cell incorporated  $1 \times 10^3$  of MM than that of cultured by the conventional 3D method (Figure 11). In cell aggregates incorporating MM, the 3D cell-cell interaction is well achieved and also the cells directly interact with matrigel. The better 3D cell-cell interaction of EpH4 cell aggregates than that of the conventional 3D method would enable cells to increase the level of  $\beta$ -casein expression. However, the reason is unclear at present. Detailed investigation on the mechanism of epithelial differentiation is required.

PI3K is a key mediator in the laminin-induced signaling cascade, which controls the activity of transcription factors essential for the gene expression of milk protein [39]. In addition, PI3K maintained the adherence junction for milk protein gene expression [34]. To investigate whether or not the PI3K is involved in the  $\beta$ -casein and E-cadherin expression of EpH4 cells, EpH4 cell aggregates with MM, GM, matrigel-coated GM

were cultured with or without LY294002 of a PI3K inhibitor [41]. The  $\beta$ -casein expression was mainly induced by prolactin/STAT5 and laminin/integrin/PI3K signaling cascade. For all the experiment groups, the  $\beta$ -casein expression was inhibited by the LY294002 addition. This indicates that the PI3K signal is required for the  $\beta$ -casein expression of EpH4 cells. Based on this, laminin present in MM would induce to promote the PI3K signaling cascade, resulting in higher  $\beta$ -casein expression. The result of this study was similar to that previously reported. In this study, for the culture without LY294002 addition, no difference in the expression of E-cadherin was observed in any experimental conditions without LY294002 addition (Figure 7). This can be explained by the aerobic metabolism of EpH4 cell aggregates. It has been demonstrated that epithelial cells often lost the E-cadherin expression by epithelial-mesenchymal transition under a hypoxic condition [42-43]. In this study, EpH4 cells in cell aggregates without or with microspheres incorporation were not under a hypoxic condition (Figures 4 and 6). Taken together, we can say certainty that the E-cadherin expression was maintained without epithelial-mesenchymal transition.

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## **Chapter 5**

### **Preparation of EpH4 and 3T3L1 cells aggregates incorporating gelatin hydrogel microspheres for a cell condition improvement**

#### **5.1 Introduction**

Epithelial-mesenchymal interactions are important and indispensable in the development of most organs, such as tooth, mammary gland, lung, kidney, and hair follicle [1–5]. The organ fundamentally develops to form from epithelium and mesenchyme by the epithelial-mesenchymal interactions during the early stage of morphogenesis. It is well recognized that the interactions are mediated by soluble paracrine factors, direct cell-cell contact, and cell-extracellular matrix (ECM) interactions [6].

Recently, epithelial and mesenchymal cells are co-cultured to investigate the mechanism of organs development [7, 8], wound healing and fibrosis [9], cancer progression and metastasis [10], and cell-based tissue and organization [11]. However, most of the researches have been performed in two dimensional (2D) systems, which is quite different from the three dimensional (3D) cell environment of living tissues. In addition, epithelial cells are not proliferated, during the culture, their polarity and functions are lost [12]. On the other hand, several 3D cell culture technologies have been reported [13–16]. Considering at the structure of body tissues, such as liver and bone, cell aggregates, physiologically work as the minimum unit of cellular function [17]. For example, embryonic stem cells generally aggregate to form an embryoid body, and consequently initiate the cell differentiation into different lineages [18]. In addition, cell aggregates produce extracellular matrix proteins more efficiently than single cells

[19]. It is possible that cell aggregation physiologically induces the cell–cell interactions, resulting in enhanced biological functions of cells. However, some technological problems still remain unsolved for the cell aggregates culture. One of the largest problems is that when as the size of cell aggregates become large, cells in the center of aggregates weaken and die. This is mainly due to the lack of oxygen and nutrients inside the aggregates. [20-21]. Another problem is difficulty to control the cells distribution for their better interaction in co-culture of epithelial and mesenchymal cells [7].

The previous study demonstrated that the incorporation of gelatin hydrogel microspheres prevented the mouse preosteoblast MC3T3-E1 cells in the aggregates suffering from a lack of oxygen and nutrient necessary for their survival because oxygen and nutrients can be permeated through the hydrogel matrix [22], resulting in a promoted their proliferation and osteogenic differentiation [23].

The objective of this study is to design a 3D cell culture technology of epithelial and mesenchymal cells aggregates for an improved epithelial cell functions and epithelial-mesenchymal interactions. This technology will be useful and available to investigate the mechanism of organs development and cell-based tissue and organization. In this study, 3D of mouse mammary epithelial EpH4 and mouse preadipocyte 3T3L1 cells in the presence of gelatin hydrogel microspheres (GM) were prepared and the effect of GM presence on the survival and functions of cells in the 3D cell aggregates were evaluated. For epithelial cells, mouse mammary epithelial EpH4 cells were used because the mammary gland provides an excellent system to investigate the hormonal regulation of specific gene expression and differentiation [24-28]. For mesenchymal cells, mouse preadipocyte 3T3L1 cells were selected. Because it is known

that the cells express high levels collagen type IV and laminin of two essential basement membrane proteins [29-30]. GM were prepared by the dehydrothermally crosslinking method, followed by treatment with fibronectin (FN) to give a cell adhesion property to the GM surface. Following, EpH4 and/or 3T3L1 cells were incubated with FN-treated GM to form their aggregates. The survival, distribution, L-lactic acid/glucose ratio (an oxygen condition measure), and  $\beta$ -casein expression of the cells aggregates in the presence of GM were evaluated comparing with those of GM-absent.

## 5.2 Materials and methods

### 5.2.1 Preparation of gelatin hydrogel microspheres

Gelatin hydrogel microspheres (GM) were prepared by the chemical cross-linking of gelatin in a water-in-oil emulsion state according to the method previously reported [31]. Briefly, an aqueous solution (20 ml) of 10 wt % gelatin (isoelectric point 5.0, weight-averaged molecular weight 1,00,000, Nitta Gelatin Inc., Osaka, Japan) was preheated at 40 °C, and then added dropwise into 600 ml of olive oil (Wako Ltd, Osaka, Japan) at 40 °C, followed by stirring at 150 rpm for 10 min to prepare a water-in-oil emulsion. The emulsion temperature was decreased to 4 °C for the gelation of gelatin solution to obtain non-crosslinked GM. The resulting microspheres were washed three times with cold acetone by centrifugation (5000 rpm, 4 °C, 5 min) to completely exclude the residual oil. Then, they were fractionated by size using sieves with apertures of 20, 32, 75, and 105 µm (Iida Seisakusyo Ltd, Osaka, Japan) and air-dried at 4 °C. The non-crosslinked and dried GM (200 mg) were treated in a vacuum oven at 140 °C and 0.1 Torr for the dehydrothermal crosslinking of gelatin for 48 hr according to the method previously reported [22]. The pictures of GM in the water-swollen and dispersed states were taken with a light microscope (CKX41, Olympus Ltd, Tokyo, Japan). For each sample, the size of 100 microspheres was measured with a computer program Image J (NIH Inc., Bethesda, USA), and the average size was calculated. For the fibronectin (FN) treatment of GM, 20 µl of 100 µg/ml FN aqueous solution (Sigma-Aldrich Inc., St. Louis, USA) was dropped onto 2 mg of freeze-dried GM, followed by leaving at 37 °C for 1 hr for adsorption of fibronectin onto the microspheres. During this treatment, it is likely that FN molecules interact with gelatin

of GM because of the bioaffinity for gelatin.

### **5.2.2 Cell culture experiments**

EpH4 cells of a mouse mammary epithelial cell line were transfected and clones selected as previously described [32]. EpH4 were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Thermo Inc., Waltham, USA) supplemented with 2 vol % fetal calf serum (FCS) (Thermo Inc., Waltham, USA), gentamicin, 3 µg/ml prolactin (Sigma-Aldrich Inc., St. Louis, USA), 1 µg/ml hydrocortisone (Sigma-Aldrich Inc., St. Louis, USA), and 5 µg/ml insulin (Sigma-Aldrich, St. Louis, USA) (standard medium), and cultured at 37 °C in a 95% air-5% carbon dioxide atmosphere. The culture medium was changed every 2 days and confluent cells were subcultured through trypsinization. In the experiment of phosphatidylinositol 3-kinase (PI3K) inhibition of EpH4 cells, the medium containing 50 µM of PI3K inhibitor LY294002 (Abcam Inc., Cambridge, UK) was used to culture for 72 hr. 3T3L1 preadipocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Inc., Carlsbad, USA) supplemented with 10 vol% FCS, penicillin (50 U/ml), and streptomycin (50 U/ml) (standard medium) and cultured at 37 °C in a 95% air-5% carbon dioxide atmosphere. The culture medium was changed every 2 days and confluent cells were subcultured through trypsinization. For the adipogenic differentiation of 3T3L1 cells, the medium supplemented using AdipoInducer Reagent (Takara-bio Inc., Shiga, Japan) including insulin, dexamethasone, and 3-isobutyl-1-methylxanthine was used according to the manufacturer's instructions.

### **5.2.3 Attachment of EpH4 cells to gelatin hydrogel microspheres with or without 5.2.4**

### ***Fibronectin-treatment***

Agarose (Sigma-Aldrich Inc., St. Louis, USA) was dissolved in phosphate buffered saline (PBS) (1 wt %). This solution (600  $\mu$ l/well) was added to each flat-bottomed well of 12-multiwell culture plate with flat bottom and incubated at 25 °C for 30 min. On the other hand, FN-treated GM and EpH4 cells were separately suspended in the standard medium. The suspensions of GM (4 mg/ml , 500  $\mu$ l/well) was added into the agarose-coated wells, and then the EpH4 cells suspension ( $1 \times 10^6$ ,  $2 \times 10^6$ ,  $4 \times 10^6$ ,  $8 \times 10^6$  cells/ml, 500  $\mu$ l/well) was added to culture at 37 °C for 6 hr. Next, the mixture of GM and EpH4 cells was passed through a cell strainers (40  $\mu$ m; Becton, Dickinson and Company Inc., Franklin Lakes, USA) to remove single EpH4 cells to collect EpH4 cells attached GM. The number of EpH4 cells attached on GM was determined by the fluorometric quantification of cellular DNA according to the method reported by Rao et al [33]. Briefly, the cells-attached GM were lysed in 30 mM sodium citrate-buffered saline solution (SSC) (pH 7.4) containing 0.2 mg/ml sodium dodecylsulfate (SDS) at 37 °C for 12 hr with occasional mixing. The cell lysate (30  $\mu$ l) was mixed with a dye solution (70  $\mu$ l; 30 mM SSC, 10  $\mu$ g/ml Hoechst 33258 dye) and the fluorescent intensity of mixed solution was measured in a fluorescence spectrometer (F-2000, HITACHI, Japan) at the excitation and emission wavelengths of 355 and 460 nm, respectively. The calibration curve between the DNA and cell number was prepared by use of cell suspensions at different cell densities. The DNA assay was done three times independently for every experimental sample unless otherwise mentioned.

#### ***5.2.4 Preparation of EpH4/3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation***

Poly(vinyl alcohol) (PVA, degree of polymerization = 1,800 and 88 mol % saponification) kindly supplied from Unichika Ltd Tokyo, Japan, was dissolved in PBS (1 wt %). This solution (100  $\mu$ l/well) was added to each round-bottomed (U-bottomed) well of a 96-multiwell culture plate and incubated at 37 °C for 15 min. Then, the solution was removed by aspiration and the wells were washed twice with PBS (100  $\mu$ l/well). For preparation of EpH4 or 3T3L1 cell aggregates without FN-treated GM, the EpH4 suspension ( $2 \times 10^5$  cells/ml, 50  $\mu$ l /well) or 3T3L1 suspension ( $2 \times 10^5$  cells/ml, 50  $\mu$ l/well) was added to the coated wells. For preparation of EpH4/3T3L1 cells aggregates without or with FN-treated GM, EpH4-cells attached and FN-treated GM and 3T3L1 cells were separately suspended in the standard medium. The suspension of FN-treated GM attached with EpH4 cells ( $1 \times 10^3$  and  $2 \times 10^4$  microspheres/ml, 50  $\mu$ l/well) were added to the coated wells, followed by the addition of 3T3L1 cells suspension ( $1 \times 10^5$  cells/ml, 50  $\mu$ l/well). On the other hand, FN-treated GM was dehydrothermally crosslinked for 48 hr, EpH4 cells, and 3T3L1 cells were separately suspended in the standard medium. The suspension of FN-treated GM (0,  $1 \times 10^3$  and  $2 \times 10^4$  microspheres/ml, 50  $\mu$ l/well) was added to the coated wells, followed by the EpH4 suspension ( $1 \times 10^5$  cells/ml, 50  $\mu$ l /well) and 3T3L1 suspension ( $1 \times 10^5$  cells/ml, 50  $\mu$ l/well). Experiments were performed on three wells for each sample unless mentioned otherwise. Photographs of cell aggregates with or without gelatin microspheres were taken with a microscope (CKX41, Olympus, Tokyo, Japan).

#### ***5.2.5 Measurement of cell number of live EpH4 and/or 3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation***

The number of live cells in EpH4/3T3L1 cells aggregates without or with FN-treated

GM was determined by counting the number of cells nuclei after their crystal violet staining [34]. Briefly, a mixed solution of 0.2 M citric acid and 0.2 wt % crystal violet was added (100  $\mu$ l/well) to each well of 96-multiwell culture plate 7 days after EpH4/3T3L1 cells were cultured without or with FN-treated GM dehydrothermally crosslinked for 48 at 140 °C. After the crystal violet staining, the cells were lysed in 0.1 wt % Triton X-100 in PBS at 37 °C overnight to separate the nuclei from the cell debris. After pipetting, the cell nuclei collected were viewed on a light microscope (CKX31-11PHP, Olympus, Tokyo, Japan) and counted in a hemocytometer (OneCell Inc., Hiroshima, Japan). The nuclei of live cells are generally round, while that of dead cells are irregularly shaped. Live cells were distinguished from dead cells by their nucleus shape.

***5.2.6 Measurement of L-lactic acid/glucose ratio of EpH4 and/or 3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation***

EpH4 and 3T3L1 cells were cultured with FN-treated GM in the similar way previously described to form the cell aggregates. The amount of glucose consumed by EpH4/3T3L1 cells aggregates was determined by measuring the change in glucose concentration in the culture medium using a Glutest Neo Super test kit (Sanwa kagaku kenkyusyo Ltd, Kyoto, Japan) 7 days after incubation. The amount of L-lactic acid produced by EpH4/3T3L1 cells aggregates was determined with an E-kit (R-Biopharm AG Co. Ltd, Germany) after 7 days incubation. The number of live cells was determined by the crystal violet staining as described above and used to normalize the amount of glucose consumption and L-lactic acid produced to the number of live cells.

The L-lactic acid/glucose ratio was calculated as a measure of aerobic condition [35].

***5.2.7 Evaluation of EpH4 and 3T3L1 cells distribution in EpH4/3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation***

To evaluate the cells distribution in cell aggregates, EpH4 cells were stained with PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich Inc., St Louis., USA), while 3T3L1 cells were stained with PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich Inc., St Louis., USA) according to the manufacturer's instructions. PKH67-labelled EpH4 cells and PKH26-labelled 3T3L1 cells were cultured with or without FN-treated GM in the similar way previously described to form the cell aggregates. After incubation for 7 days, the aggregates were fixed with 4 wt % paraformaldehyde at 4 °C for 1 hr and embedded in an optimal cutting temperature compound (Sakura Finetek Japan Co. Ltd, Tokyo, Japan) and frozen using liquid nitrogen. The frozen samples were sectioned using a cryotome (CM3050S, Leica Microsystems, Wetzlar Inc., Germany). The sections of 10 µm thickness were viewed in a confocal laser scanning microscope (FV1000D, Olympus Ltd, Tokyo, Japan) to evaluate the cells distribution in aggregates.

***5.2.8 Immunostaining of  $\beta$ -casein and laminin expression of EpH4/3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation***

Eph4/3T3L1 cells aggregates with or without FN-treated GM were prepared. After incubation for 7 days, the aggregates were fixed with 4 wt % paraformaldehyde at 4 °C for 1 hr and embedded by using an optimal cutting temperature (O.C.T) compound

(Sakura Finetek Japan Ltd, Tokyo, Japan) and frozen using liquid nitrogen. The frozen samples were sectioned using a cryotome (CM3050S, Leica Microsystems, Wetzlar Inc., Germany) and incubated at 4 °C overnight with the following primary antibody against:  $\beta$ -casein (Santacruz Inc., America, USA, 1:50) or laminin (Abcam Inc., Cambridge, UK, 1:100). Then, a secondary antibody coupled to Alexa 488 (Molecular Probes, Invitrogen Inc., USA, 1:700) was incubated at 25 °C for 30 min to immunologically stain the corresponding protein. Next, TO-PRO-3 (Molecular Probes, Eugene Inc., USA) was incubated at 25 °C for 10 min for the nuclear detection. The sections of 10  $\mu$ m thickness were viewed in a confocal laser scanning microscope (FV1000D, Olympus Ltd, Tokyo, Japan).

### ***5.2.9 Measurement of $\beta$ -casein expression of EpH4/3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation***

The messenger ribonucleic acid (mRNA) expression of  $\beta$ -casein was evaluated by the conventional real-time polymerase chain reaction (PCR). Briefly, EpH4 and/or 3T3L1 cells aggregates with or without FN-treated GM were prepared similarly. After incubation for 7 days, RNA was extracted and cleaned up using the Qiagen RNeasy® Plus Mini kit (Qiagen Inc., Germany) according to the manufacturer's instructions. The total RNA sample was reverse-transcribed to cDNA using SuperScript VILO™ (Invitrogen Inc., Carlsbad, CA). The resulting cDNA was subjected to the real-time PCR assay by using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Inc., Carlsbad, CA) to determine the gene expression of  $\beta$ -casein. The PCR reaction was carried out with specific primers in the presence of Power SYBR Green (Applied Biosystems Inc., Carlsbad, CA) as described in the manufacturer's instruction.

The level of gene expression was normalized by that of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, and calculated as the ratio to EpH4 cell culture without GM as a control group. Experiments were performed for 5 specimens independently for each sample. The primer sequences for GAPDH and  $\beta$ -casein were as follows, GAPDH: 5-TGAAGCAGGCATCTGAGGG -3, 5-CGAAGGTGGAAGAGTGGGAG-3;  $\beta$ -casein: 5- GGTGAATCTCATGGGACAGC-3, 5-TGACTGGATGGTGGAGTGAA-3.

#### **5.2.10 Statistical analysis**

All the statistical data are expressed as the mean $\pm$ standard error of the mean (SEM). The data were analyzed by t-test to determine the statistical significance of differences between two mean values, which was accepted at a p value of  $<0.05$ .

## 5.3 Results

### 5.3.1 Characterization of gelatin hydrogel microspheres

Figure 1 shows typical microscopic pictures of gelatin hydrogel microspheres (GM). The microspheres were of spherical shape and had a smooth surface. The sizes of GM prepared in the water-swollen condition were  $50.1 \pm 18.3$  and  $194.3 \pm 33.0$   $\mu\text{m}$ .

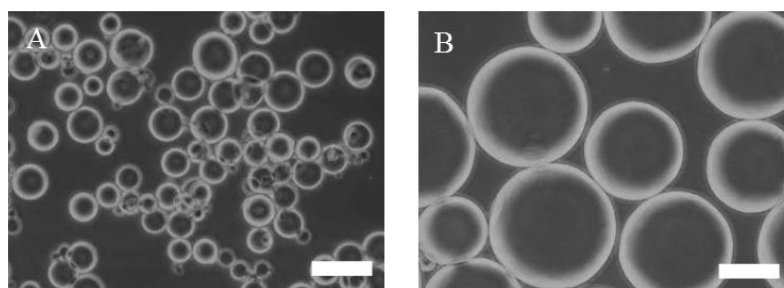


Figure 1. Light microscopic pictures of gelatin microspheres with diameters of  $50.1 \pm 18.3$   $\mu\text{m}$  (GM50) and  $194.3 \pm 33.0$   $\mu\text{m}$  (GM200) dispersed in water. Scale bar. 100  $\mu\text{m}$ .

### 5.3.2 Number of EpH4 cells attached onto gelatin hydrogel microspheres without or with treatment of fibronectin

Figure 2 shows the number of cells attached onto FN-treated GM50 and GM200 dispersed in water. The number of cells attached to FN-treated GM50 was about 5, while that of cells attached to FN-treated GM200 was about 100. On the other hand, the number of cells was less than 1 for GM50 and GM200 without treatment of FN (data not shown). The EpH4 adhesion to GM was improved by the treatment of FN, and a higher number of EpH4 cells attached onto GM was observed for FN-treated GM200.

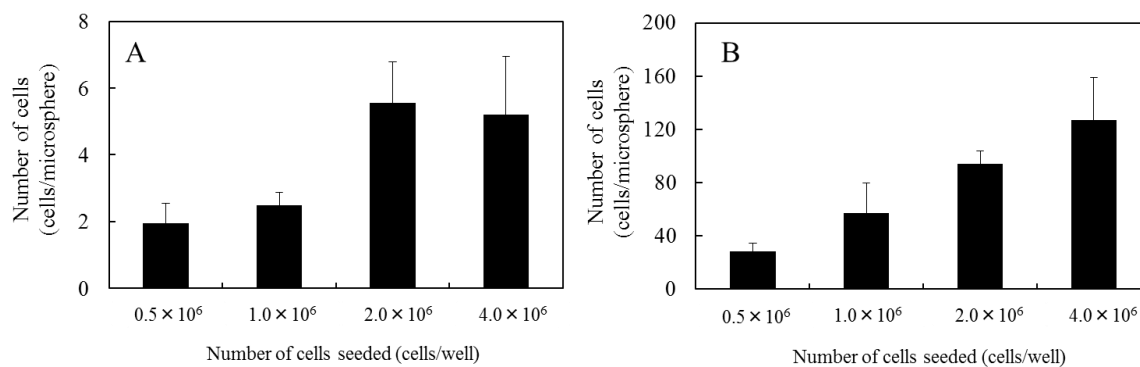


Figure 2. The number of EpH4 cells attached onto gelatin microspheres dispersed in water. The number of cells was about 5 for GM50 (A) and the number of cells attached was about 100 for GM200 (B).

### 5.3.3 Formation of EpH4 and/or 3T3L1 cell aggregates incorporating fibronectin-treated gelatin hydrogel microspheres

EpH4 and/or 3T3L1 cells aggregates without or with FN-treated GM were formed in different culture conditions (as shown in Figure 3).

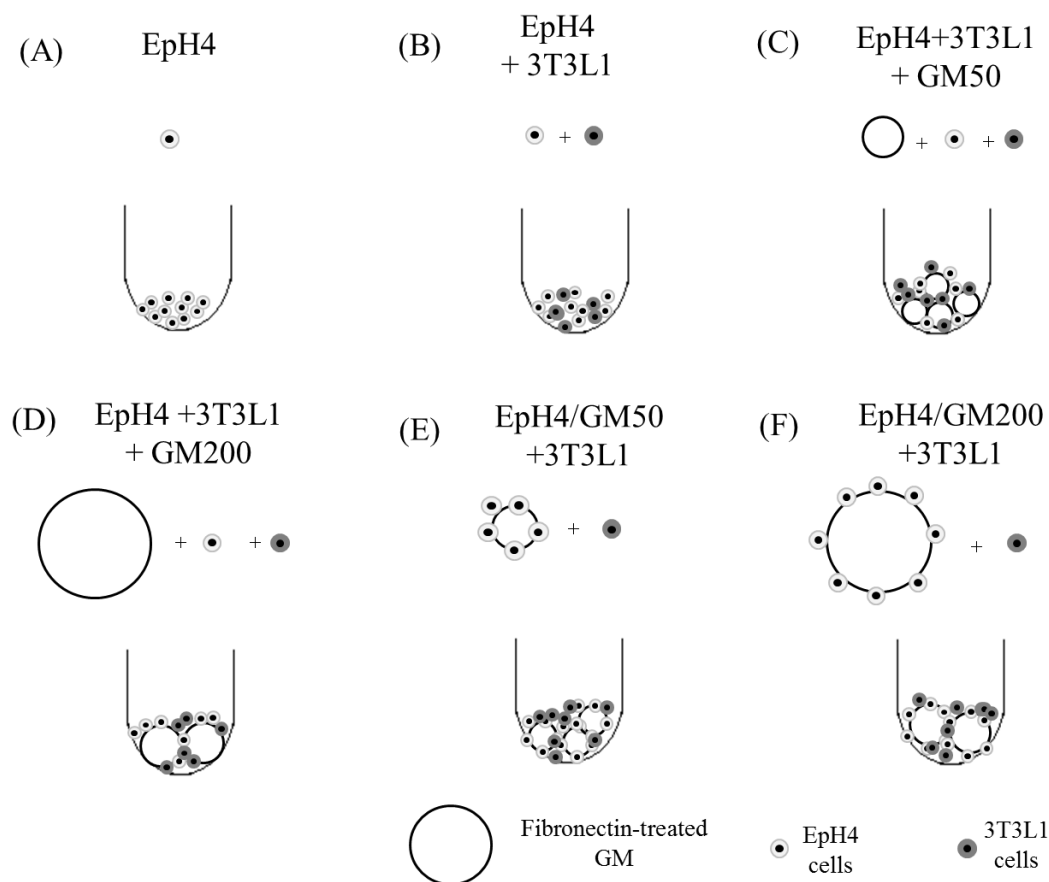


Figure 3. Experimental conditions of EpH4 and/or 3T3-L1 cells cultured with or without FN-treated GM; EpH4 cells without GM (A), EpH4 and 3T3L1 cells aggregates without GM (B), EpH4 and 3T3L1 cells and FN-treated GM50 (C), EpH4 and 3T3L1 cells and FN-treated GM200 (D), EpH4 cells precultured with GM50 and 3T3L1 cells (E), and EpH4 cells precultured with GM200 and 3T3L1 cells (F).

Figure 4 shows microscopic pictures of EpH4 and/or 3T3L1 cell aggregates 1, 3, 5, and 7 days after incubation without FN-treated GM. For 3T3L1 cells only or mixture of EpH4 and 3T3L1 cells, the cells aggregates were rapidly formed and their shape was spherical. On the contrary, aggregates of EpH4 cells only were formed slowly and not of

spherical shape.

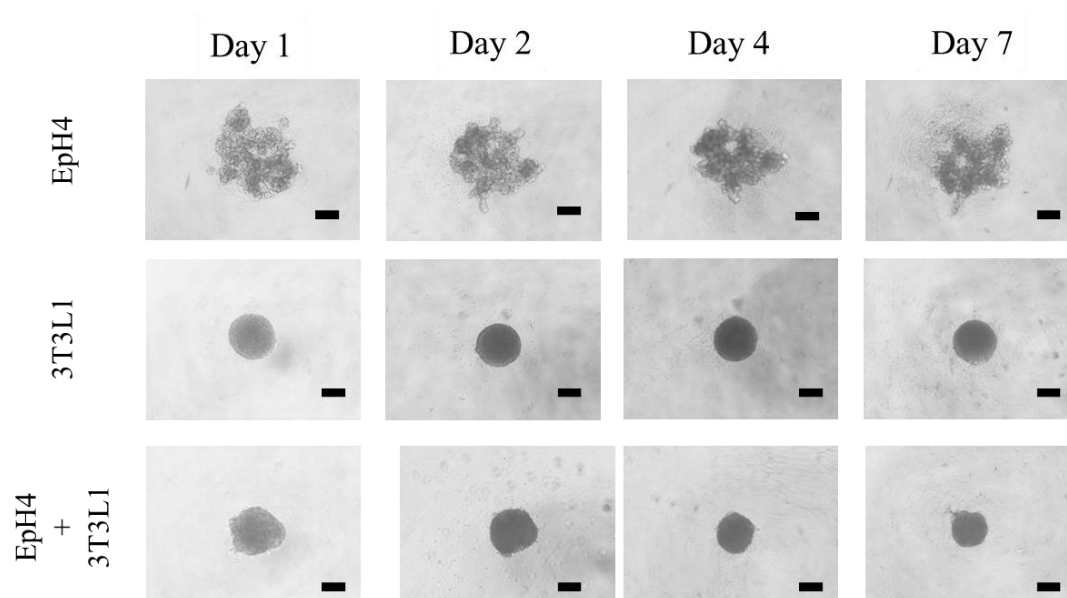


Figure 4. Light microscopic pictures of EpH4 and/or 3T3L1 cells aggregate 1, 3, 5, and 7 days after incubation without FN-treated GM. Scale bar. 100  $\mu$ m.

Figure 5 shows microscopic pictures of EpH4 and 3T3L1 cells aggregates in the presence FN-treated GM with or without EpH4 cells pre-incubation 7 days after incubation. In all cases, the EpH4/3T3L1 cells aggregates with both types of FN-treated GM were formed.

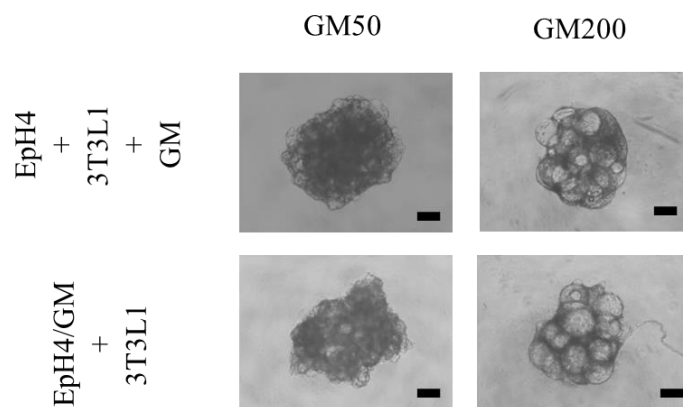


Figure 5. Light microscopic pictures of EpH4 cells preincubated with FN-treated GM50 and GM200 and 3T3L1 cells 7 days after incubation. Scale bar. 100  $\mu$ m.

#### 5.3.4 Number of live cells in EpH4 and/or 3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation

Figure 6 shows the number of live EpH4 and 3T3L1 cells in EpH4/3T3L1 cells aggregates 7 days after incubation with or without FN-treated GM. The larger number of live EpH4 and 3T3L1 cells was observed for EpH4/3T3L1 cell aggregates with FN-treated GM, irrespective of their size and EpH4-preincubation. The number of live EpH4 and 3T3L1 cells in EpH4/3T3L1 cells aggregates was influenced by the incorporation of FN-treated GM to aggregates.

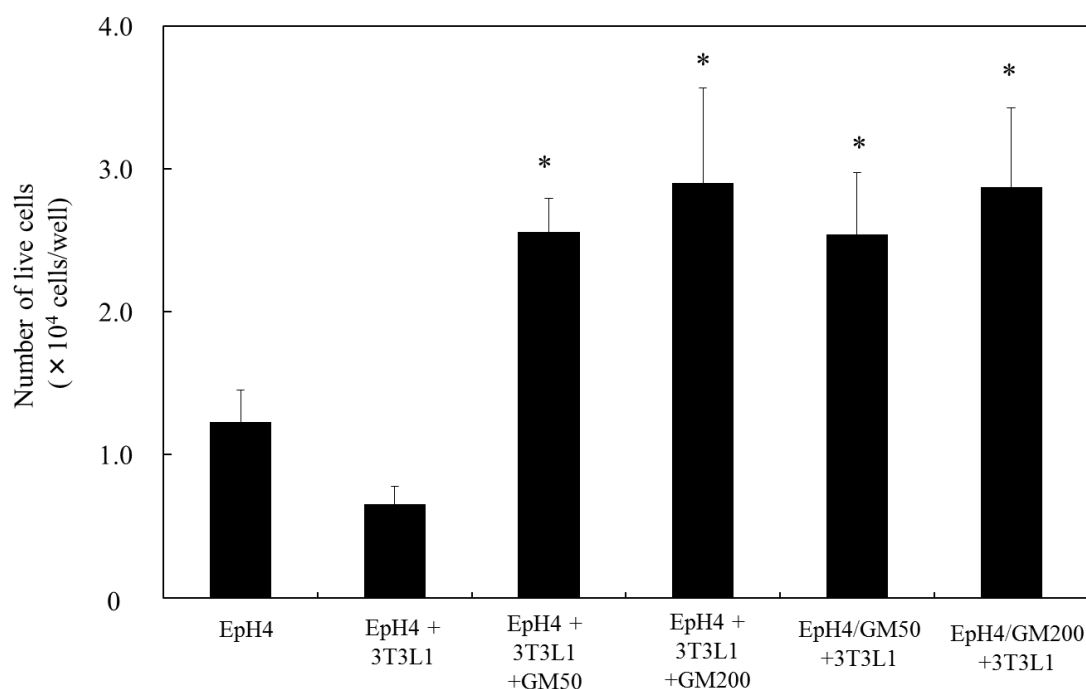


Figure 6. Number of live EpH4 and 3T3L1 cells in EpH4 and/or 3T3L1 cells aggregates with or without FN-treated GM50 7 days after incubation.

\*  $p < 0.05$ , significant against the number of live EpH4 and 3T3L1 cells in EpH4/3T3L1 aggregates cultured without GM.

### 5.3.5 L-lactic acid/ glucose ratio of EpH4 and/or 3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation

Figure 7 shows the L-lactic acid/glucose ratio of EpH4 and 3T3L1 cells in EpH4/3T3L1 cells aggregates 7 days after incubation with or without FN-treated GM. The higher L-lactic acid/glucose ratio of EpH4 and 3T3L1 cells was observed for EpH4/3T3L1 cells aggregates without FN-treated GM. The L-lactic acid/glucose ratio of EpH4 and 3T3L1 cells aggregates decreased to a significantly great extent by the incorporation of GM50 and GM200. The L-lactic acid/glucose ratio of EpH4 and

3T3L1 cells in EpH4/3T3L1 cells aggregates were greatly influenced by the incorporation of FN-treated GM to aggregates.

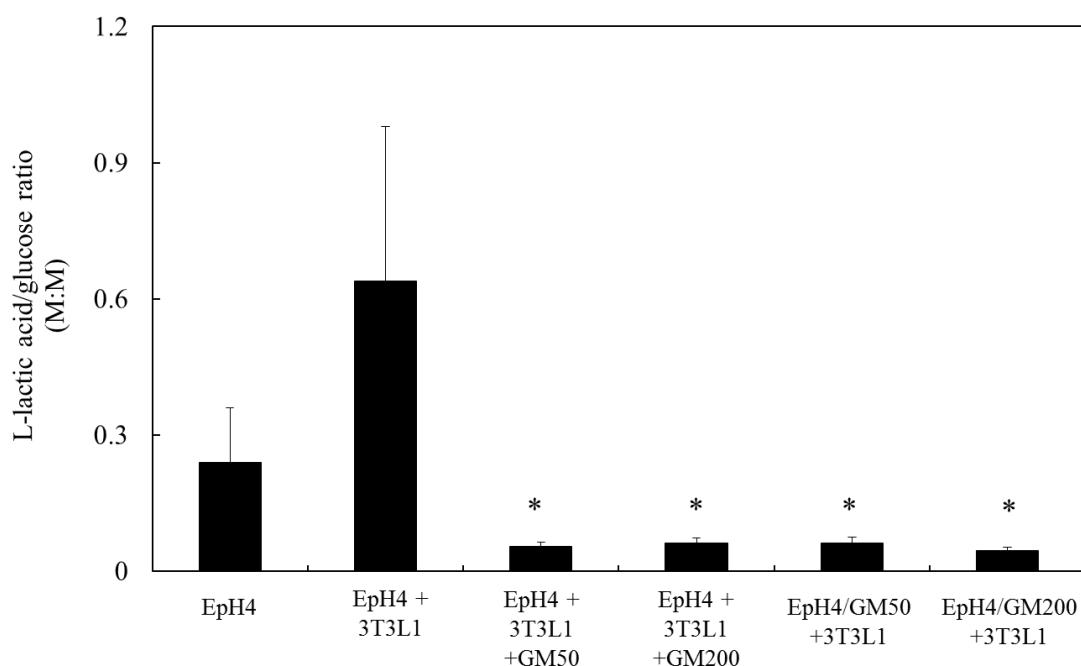


Figure 7. L-lactic acid/glucose ratio of EpH4 and 3T3-L1 cells in EpH4 and/or 3T3-L1 cell aggregates with or without FN-treated GM 7 days after incubation \*  $p < 0.05$ , significant against the L-lactic acid/glucose ratio of EpH4 cells in EpH4/3T3L1 aggregates cultured without GM.

### 5.3.6 Distribution of EpH4 and 3T3L1 cells in EpH4/3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation

Figure 8 shows the confocal laser microscopic pictures of EpH4 and 3T3L1 cells in EpH4/3T3L1 cells aggregates 7 days after incubation with or without FN-treated GM. For the EpH4/3T3L1 cells aggregates without FN-treated GM, EpH4/3T3L1 cells aggregates showed a core-shell structure where the 3T3L1 cells and EpH4 cells were

localized inside and outside of EpH4/3T3L1 cell aggregates respectively. On the contrary, for the EpH4/3T3L1 cells aggregates with FN-treated GM, the EpH4 and 3T3-L1 cells homogeneously distributed in the EpH4/3T3L1 cells aggregates. However, 3T3L1 cells tended to localize inside of EpH4/3T3L1 cells aggregates incorporating FN-treated GM.

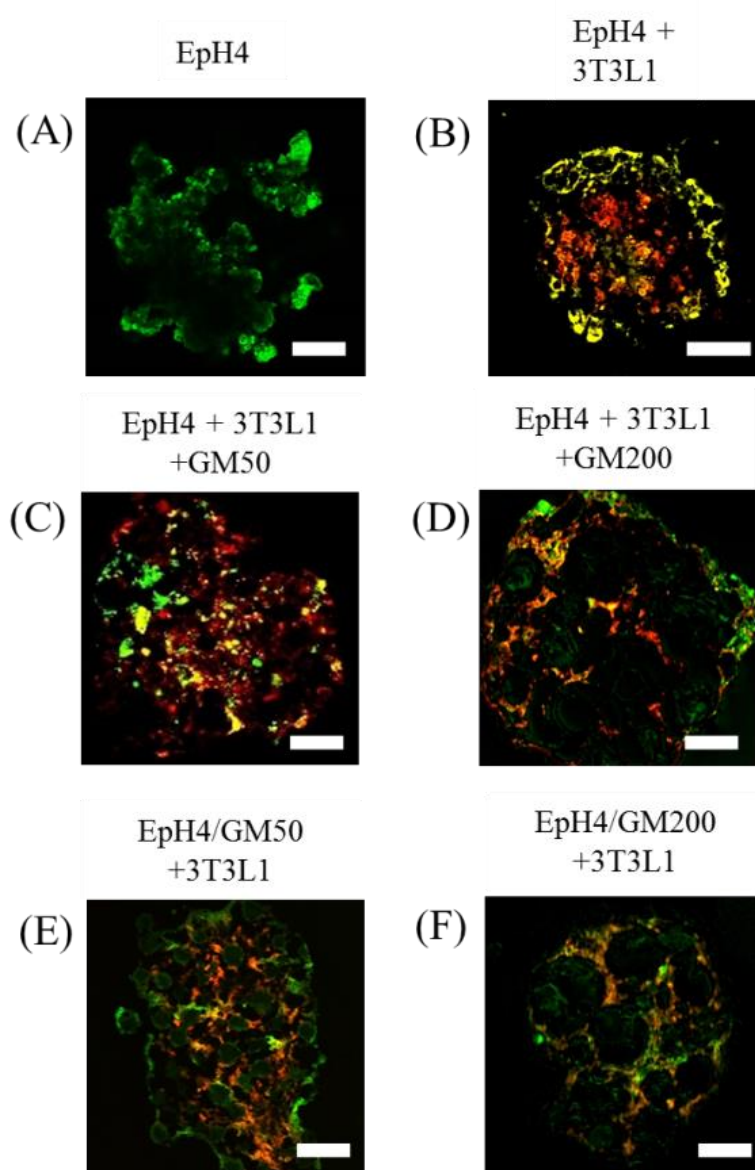


Figure 8. Fluorescent microscopic pictures of EpH4 and 3T3-L1 cells in

EpH4/3T3L1 cells aggregates 7 days after incubation with or without FN-treated GM; EpH4 cells without gelatin microspheres (A), EpH4 and 3T3L1 cells aggregates without gelatin microspheres (B), EpH4 and 3T3L1 cells and FN-treated GM50 (C), EpH4 and 3T3L1 cells and FN-treated GM200 (D), EpH4 cells precultured with GM50 and 3T3L1 cells (E), and EpH4 cells precultured with GM200 and 3T3L1 cells (F). Scale bar. 100  $\mu$ m.

***5.3.7 Expression of  $\beta$ -casein and laminin of EpH4/3T3L1 cells aggregates without or with fibronectin-treated gelatin hydrogel microspheres incorporation***

Figures 9 and 10 show the confocal laser microscopic pictures of laminin and  $\beta$ -casein expression of EpH4 cells in EpH4/3T3L1 cells aggregates 7 days after incubation with or without FN-treated GM. The higher laminin and  $\beta$ -casein expressions were observed for EpH4/3T3L1 cells aggregates with FN-treated GM than those of GM-absent EpH4/3T3L1 cells aggregates.

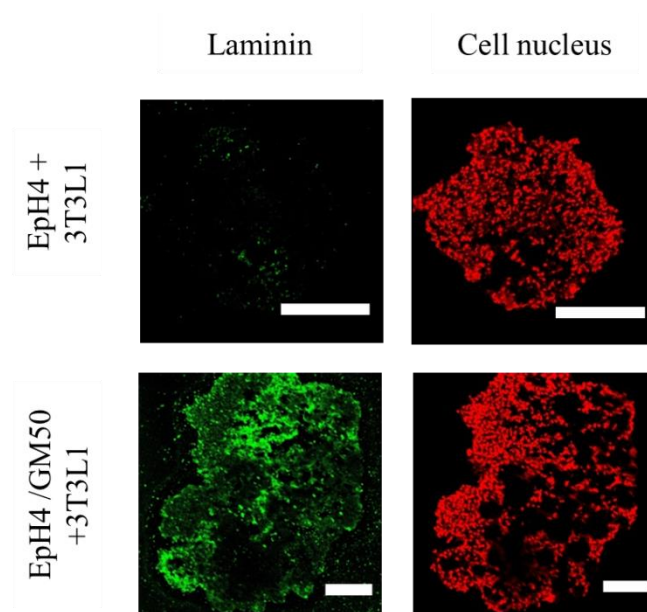


Figure 9. Fluorescent microscopic pictures of laminin expression in EpH4/3T3L1 cells aggregates with or without FN-treated GM50 7 days after incubation. Scale bar. 100  $\mu$ m.

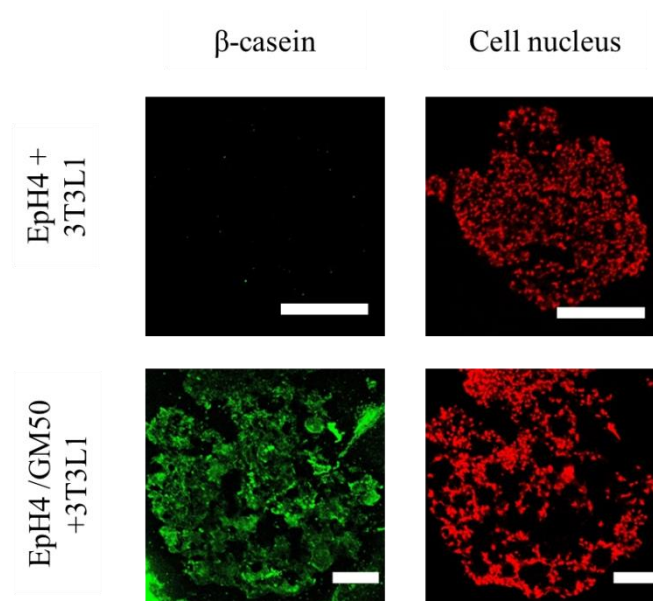


Figure 10. Fluorescent microscopic pictures of  $\beta$ -casein expression in EpH4/3T3L1 cells aggregates with or without FN-treated GM50 7 days

after incubation. Scale bar. 100  $\mu$ m.

Figure 11 shows the  $\beta$ -casein mRNA expression of EpH4 cells in EpH4/3T3L1 cells aggregates 4, 7, and 14 days after incubation with or without FN-treated GM. The higher  $\beta$ -casein expression was observed for EpH4/3T3L1 cells aggregates with FN-treated GM than that of without FN-treated GM even after a long-term culture of 14 days. A higher  $\beta$ -casein expression was observed for EpH4/3T3L1 cells aggregates with FN-treated GM50 than that of aggregates with GM200 added at the same time. When EpH4 cells, 3T3L1 cells, and GM were added at the same time, the  $\beta$ -casein expression was high compared with that of 3T3L1 cells cultured with GM-preincubated EpH4 cells. The laminin and  $\beta$ -casein expressions of EpH4 and 3T3L1 cells in EpH4/3T3L1 cells aggregates were influenced by the incorporation of FN-treated GM to aggregates. In addition, the  $\beta$ -casein expression of EpH4 cells in EpH4/3T3L1 cells aggregates was modified by the size of FN-treated GM.

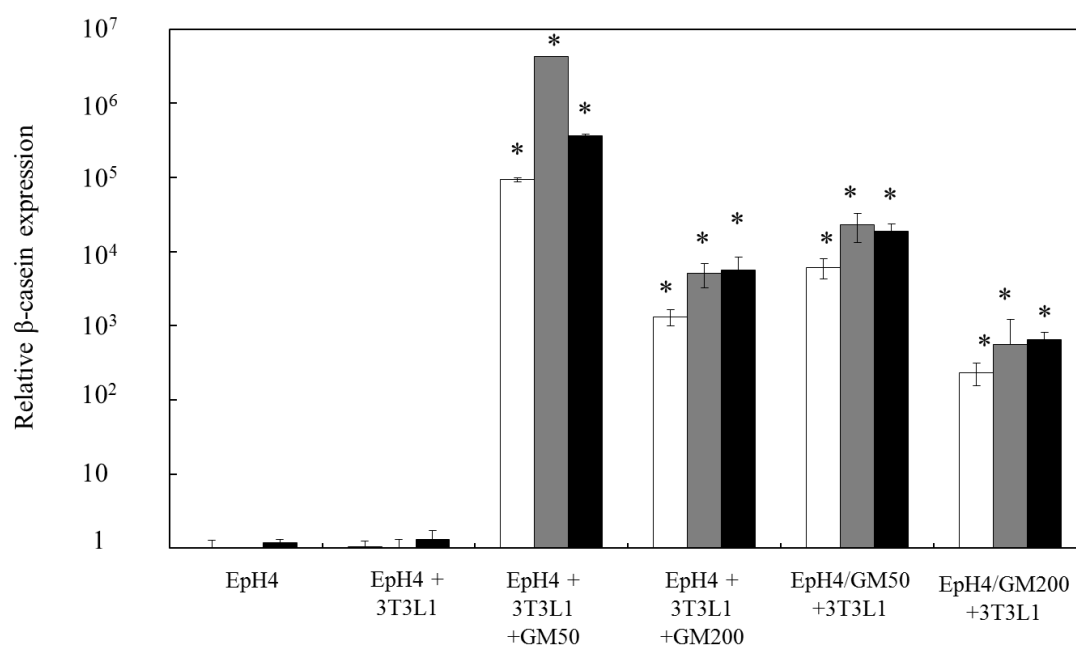


Figure 11.  $\beta$ -casein mRNA expression of EpH4 cells in EpH4/3T3L1 cells aggregates with or without FN-treated GM50 and GM200 4 (□), 7 (■), and 14 (■) days after incubation. \*  $p < 0.05$ , significant against the  $\beta$ -casein mRNA expression of EpH4 cells in EpH4/3T3L1 cells aggregates without GM at the corresponding incubation time.

Figure 12 shows the effect of LY294002 addition on the  $\beta$ -casein mRNA expression of EpH4 cells in EpH4/3T3L1 cells aggregates 7 days after incubation with or without FN-treated GM. Irrespective of the FN-treated GM size and the incubation manner, the  $\beta$ -casein expression was inhibited by the addition of LY294002.

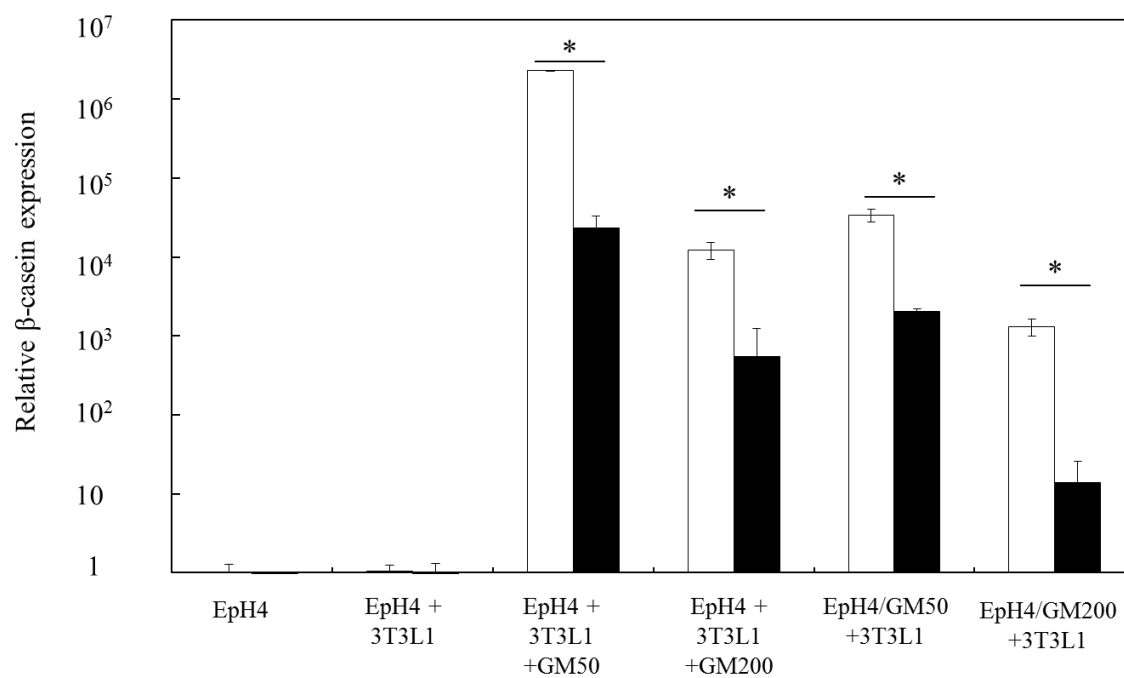


Figure 12.  $\beta$ -casein mRNA expression of EpH4 cells in EpH4/3T3L1 cells aggregates with or without FN-treated GM50 and GM200 7 days after incubation in the absence ( $\square$ ) and presence ( $\blacksquare$ ) of LY294002. \*  $p < 0.05$ , significant between two groups.

## 5.4 Discussion

Gelatin hydrogel microspheres were fractionated by using size sieves to obtain the different sizes of microspheres in the water dispersed state (Figure 1). The GM were treated by fibronectin (FN) because it allowed the microspheres to enhance their EpH4 cell attachment. It is known that FN is one of the cell adhesion molecules [36]. Less number of EpH4 cells was attached to GM without treatment of FN (data not shown). For FN-treated GM, as expected, the FN-treated GM were susceptible to the attachment of the larger number of EpH4 cells attached onto GM with the larger size (Figure 2). It is possible that the surface area of GM increase with an increase in their size, resulting in larger number of EpH4 cells attached.

For the formation of EpH4/3T3L1 cells aggregates without FN-treated GM, the aggregates of 3T3L1 cells only and EpH4/3T3L1 cells were rapidly formed and the shape was spherical. However, for EpH4 cells only, the cell aggregates were formed slowly and the shape is not spherical (Figure 4). This may be explained in terms of cells junctions and movement. It has been demonstrated that the movement of epithelial cells is lower than that of mesenchymal cells because epithelial cells has strong cell-cell junctions of tight junctions, gap junctions, and adherens junctions [37-39]. In this study, it is conceivable that movement rate of mesenchymal 3T3L1 cells was high compared with that of epithelial EpH4 cells because of the strong epithelial cells junctions. The difference in the cells junctions and movement may result in the formation of non-spherical aggregates.

The inside localization of 3T3L1 cells and outside distribution of EpH4 cells were observed for in EpH4/3T3L1 cells aggregates without the GM (Figure 8). It has been reported that mesenchymal 3T3-L1 cells have a higher mobility and self-organization

potential than epithelial EpH4 cells. In this study, 3T3L1 cells were initially aggregated, followed by the distribution of EpH4 cells outside of 3T3-L1 cell aggregates. When human aortic fibroblast (HAF) cells were co-cultured with human umbilical vein endothelial cells (HUVEC), HAF cells were localized inside in HUVEC/HAF cells aggregates because HAF cells has a higher self-organization potential than HUVEC [40-42]. On the other hand, for the EpH4/3T3L1 cells aggregates with FN-treated GM, the EpH4 and 3T3L1 cells were homogeneously distributed in the EpH4/3T3L1 cells aggregates (Figures 5 and 8). It is reported that cells-microspheres interactions were important for the formation of cell aggregates incorporating microspheres. For example, a larger number of microspheres in pluripotent stem cells-microspheres aggregates was present for gelatin microspheres than agarose microspheres because gelatin has higher cell adhesive properties than agarose for pluripotent stem cell [40]. It is highly conceivable that FM-treated GM functioned as a scaffold of both 3T3L1 and EpH4 cells, resulting in the inhibition of initial 3T3L1 self-organization and the consequent homogeneous distribution of EpH4 and 3T3L1 cells in EpH4/3T3L1 cells aggregates.

The ratio of L-lactic acid production to glucose consumption is a general measure of aerobic metabolism [43]. The lower the ratio is, the higher the aerobic metabolism of cells aggregates is. It is apparent from Figure 7 that the lactic acid/glucose ratio of EpH4/3T3L1 cells aggregates cultured with FN-treated GM was significantly lower than that of aggregates without GM. We can say with certainty that the homogeneous incorporation of FN-treated GM into EpH4/3T3L1 cells aggregates increases the oxygen level, resulting in a lower ratio of L-lactic acid to glucose, in other words, the achievement of an aerobic culture condition. In addition, the presence of gelatin hydrogel microspheres distributed in the cell aggregate may increase oxygen permeation

inside the aggregates because oxygen can be permeated through the water phase of hydrogel matrix [22]. As a results, in case of EpH4/3T3L1 cells aggregates with FN-treated GM, the larger number of live EpH4 and 3T3L1 cells in the cell aggregate incorporating GM was observed (Figure 6). This may be because the presence of GM increases oxygen and nutrients permeability through the aggregate.

Preadipocyte 3T3L1 cells express the high levels of two essential basement membrane proteins, collagen type IV and laminin [29, 30]. The  $\beta$ -casein expression of EpH4 cells is required to allow the 3T3L1 cells to produce the basement membrane proteins collagen type IV and laminin [44-46]. It has been demonstrated that the laminin regulates the basal localization and activation of PI3K to sustain the STAT5 activation, followed by the  $\beta$ -casein expression [47-49]. The higher laminin and  $\beta$ -casein expression were observed for EpH4/3T3L1 cells aggregates with FN-treated GM (Figures 9, 10, and 11). In case of GM-absent EpH4/3T3L1 cells aggregates, 3T3L1 cells were localized inside EpH4/3T3L1 cells aggregates, but did not always function because of the lack of oxygen. On the other hand, for the GM-present EpH4/3T3L1 cells aggregates, 3T3L1 cells in EpH4/3T3L1 cell aggregates well functioned to secrete laminin due to better oxygen conditions, resulting in better EpH4-3T3L1 cells interactions mediated by laminin secreted from 3T3L1 cells. Consequently, the  $\beta$ -casein expression of EpH4 cells would become higher. The higher  $\beta$ -casein expressions were observed for EpH4/3T3L1 cells aggregates with FN-treated GM50 than that of GM200. It is likely that the larger GM would physically impair aggregates with interaction between EpH4 and 3T3L1 cells. The mixing way of EpH4 cells, 3T3L1 cells, and GM also affected the level of  $\beta$ -casein expression (Figure 8). Less contact of EpH4 cells with 3T3L1 cells was detected in EpH4/3T3L1 cells aggregates formed from 3T3L1

cells and EpH4 cells-attached GM. This may lead to a weaker EpH4-3T3L1 cells interactions and the consequent lower  $\beta$ -casein expression.

The LY294002 is known as a PI3K inhibitor [50]. To investigate the involvement of PI3K signaling pathway in the  $\beta$ -casein expression of EpH4 cells, the formation of EpH4/3T3L1 cells aggregates incorporating FN-treated GM was evaluated in the presence and absence of LY294002. As a result, the  $\beta$ -casein expression was inhibited by the LY294002 addition (Figure 12). This findings indicates that the PI3K signaling pathway is required for the  $\beta$ -casein expression of EpH4 cells. It is reported that laminin regulates the PI3K basal localization and subsequent  $\beta$ -casein expression of EpH4 cells, but LY294002 did not completely inhibit the  $\beta$ -casein expression. This is because there was another prolactin receptor-STAT5 signaling pathway for the  $\beta$ -casein expression [48]. This pathway also contribute to this phenomenon. This phenomenon obtained in this study is similar to the results previously reported.

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## **Summary**

### **Chapter 1**

When MC3T3-E1 cells were co-cultured with GM of different sizes in PVA-coated U-bottomed wells, cell aggregates incorporating GM homogeneously distributed were formed. Comparing at the same surface of GM used, the L-lactic acid/glucose ratio and the cell density of cell aggregates were significantly lower for the GM of larger sizes than those of smaller sizes. In addition, the higher ALP activity and calcium content of cell aggregates incorporating GM were observed for the GM of larger sizes. For a long-term culture, the incorporation of GM of larger sizes gave a better oxygen and nutrients supply to cells in the aggregates, resulting in an enhanced osteogenic differentiation of cells.

### **Chapter 2**

Upon co-culturing MC3T3-E1 cells with the GM in the PVA-coated round U-bottom wells, cell aggregates with GM homogeneously distributed were formed. The longer time period remaining of slow-degraded GM in the cell aggregates at large amounts resulted in higher proliferation of MC3T3-E1 cells. The ratio of L-lactic acid production/glucose consumption of cell aggregates was significantly lower than that of aggregates incorporating fast-degraded ones. The ALP activity and calcium content of MC3T3-E1 cells were higher for the cell aggregates incorporated slow-degraded GM. It is possible that the incorporation of GM gave cells in the aggregate better culture conditions, resulting in improved their survival, proliferation, and osteogenic differentiation.

### Chapter 3

Following the co-culture of MC3T3-E1 cells with GM-BMP2 in PVA-coated U-bottomed wells, cell aggregates incorporating GM-BMP2 were formed. When MC3T3-E1 cells cultured with GM-BMP2 or free BMP2 for 7 days, the higher RUNX2 mRNA expression and ALP activity were observed for MC3T3-E1 cell aggregates incorporating the fast-degraded GM-BMP2 than free BMP2 added into the medium. After 21 days culture, the high ALP activity and calcium content were observed for the medium-degraded GM-BMP2 compared with other experimental groups. The BMP2 of GM-BMP2 incorporated in the cell aggregates enhanced the osteogenic differentiation of cells compared with free BMP2 added externally. The degradability of GM-BMP2 affected the extent of osteogenic differentiation.

### Chapter 4

When EpH4 cells were co-cultured with MM in PVA-coated U-bottomed wells, cell aggregates were formed with MM homogeneously distributed. The EpH4 cells proliferation was high for EpH4 cell aggregates incorporating MM compared with the conventional 3D culture method. The expression of  $\beta$ -casein expression as a measurement of epithelial differentiation was higher for EpH4 cell aggregates incorporating MM than that of the conventional 3D culture method. The incorporation into EpH4 cell aggregates is promising to promote the proliferation and differentiation of EpH4 cells in aggregates.

### Chapter 5

Upon co-culturing FN-treated GM, EpH4 cells, and 3T3L1 cells in PVA-coated U-bottomed wells, EpH4/3T3L1 cells aggregates with FN-treated GM were formed. The higher  $\beta$ -casein expression level of EpH4 cells in EpH4/3T3L1 cells aggregates in the presence of GM was observed than that of cells in the absence of GM. Laminin secretion was also promoted for the EpH4/3T3L1 cells aggregates cultured with FN-treated GM. The presence of FN-treated GM in the EpH4/3T3L1 cells aggregates gave a better condition to the cells in the aggregates, resulting in an enhanced generation of  $\beta$ -casein from EpH4 cells in the aggregates.



## **List of publications**

Chapter 1. Shuhei Tajima, Yasuhiko Tabata.; Preparation of cell aggregates incorporating gelatin hydrogel microspheres with different sizes. Submitted (2017).

Chapter 2. Shuhei Tajima, Yasuhiko Tabata.; Preparation and functional evaluation of cell aggregates incorporating gelatin microspheres with different degradabilities. J Tissue Eng Regen Med 10: 801-811 (2013).

Chapter 3. Shuhei Tajima, Yasuhiko Tabata.; Preparation of cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities. Submitted (2017).

Chapter 4. Shuhei Tajima, Yasuhiko Tabata.; Preparation of epithelial cell aggregates incorporating matrigel microspheres to enhance proliferation and differentiation of epithelial cells. Regenerative Therapy. In press (2017).

Chapter 5. Shuhei Tajima, Yasuhiko Tabata.; Preparation of EpH4 and 3T3L1 cells aggregates incorporating gelatin hydrogel microspheres for a cell condition improvement. Regenerative Therapy. In press (2017).

## **Other publications**

Kohara H, Tajima S, Yamamoto M, Tabata Y. ; Angiogenesis induced by controlled release of neuropeptide substance P. *Biomaterials.*, 31(33): 8617-25 (2010).

Konishi S, Teramachi Y, Shimomura S, Tonomura W, Tajima S, Tabata Y. ; Cellular aggregate capture by fluidic manipulation device highly compatible with micro-well-plates. *Biomed Microdevices.*, 17(3): 9953 (2015).

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Konishi S, Shimomura S, Tajima S, Tabata Y. ; Implementation of soft microfingers for a hMSC aggregate manipulation system. *Microsystems & Nanoengineering.*, 2, 15048 (2016).

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