Preparation of two- and three-dimensional substrates with different surface properties to culture human fat-derived stem cells

Sachiko Inoue

2008

CONTENTS

PART I   CULTURE OF HUMAN FAT-DERIVED STEM CELLS ON TWO-DIMENSIONAL SUBSTRATES WITH DIFFERENT SURFACE PROPERTIES

Chapter 1. Proliferation and differentiation of human fat-derived stem cells on substrates with different water contact angles

Chapter 2. Proliferation and adipoigenic differentiation of human fat-derived stem cells on substrates modified with SAM

Chapter 3. Adipogenic differentiation of human fat-derived stem cells cultured with bFGF in the solution and coated form

Chapter 4. Adhesion of human fat-derived stem cells on substrates modified with RGD peptide

PART II   CULTURE OF HUMAN FAT-DERIVED STEM CELLS ON THREE-DIMENSIONAL SUBSTRATES WITH DIFFERENT SURFACE PROPERTIES

Chapter 5. Proliferation and osteogenic differentiation of human fat-derived stem cells on PET non-woven fabrics by different culture methods

Chapter 6. Adhesion and proliferation of human fat-derived stem cells on two- and three-dimensional PET substrates modified with RGD peptide

ABBREVIATIONS

ALP	Alkaline phosphate
aP2	Fatty acid binding protein 2
bFGF	Basic fibroblast growth factor
DDW	Double-distilled water
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
F-actin	Flamentous actin
FAK	Forcal adhesion kinase
GPDH	Glycerol-3-phosphate dehydrogenase
MSC	Mesenchymal stem cell
PBS	Phosphate-buffer saline solution
PET	Poly(ethylene terephthalete)
PPARγ2	Peroxisome proliferator-activated receptor γ2
RGD	Arg-Gly-Asp
RT-PCR	Reverse transcription polymerase chain reaction
SAM	Self-assembled monolayers

GENERAL INTRODUCTION

It is recently recognized that cells isolated from tissues in the adult body have an inherent potential of proliferation and differentiation into various lineages of cells [1, 2]. The cells are called as adult stem cells. It is expected that the potential is useful to induce cell-based tissue regeneration. For example, it has been experimentally demonstrated that stem cells transplanted to a bone defect can differentiate into osteogenic cells and assist to induce bone regeneration at the defect [3]. In addition, since the adult stem cells can be isolated from patients themselves, there are no clinical problems, such as the immunological rejection, the clinical ethics, and the shortage of cell donor. The adult stem cells used most frequently are MSC which can be isolated from the bone marrow. MSC have their high potentials of proliferation and differentiation into osteogenic, adipogenic, chondrogeni, myogenic, and neurogneic cells [4, 5]. Although the MSC have been widely investigated for the clinical applications and used for some clinical trials, there are some problems to be resolved, including the patients' pain and the technical difficulty in cell isolation, and the low number and poor differentiation potentials of cells prepared from the bone marrow of aged patients. On the other hand, fat-derived stem cells can be isolated from the fat tissue [6-8]. It is recognized that the biological properties are similar to those of MSC [6]. The isolation procedure of fat-derived stem cells is clinically easier than that of MSC, while the fat tissue is often removed for cosmetic surgery. However, the basic researches about fat-derived stem cells are scientifically delayed compared with those of MSC, although they are clinically applicable. Therefore, it is necessary to develop the technology to allow the cells to effectively proliferate and differentiate for the clinical applications.

Generally, substrates for cell culture are important for the adhesion of cells, and the subsequent proliferation and differentiation, because it is known that most of cells cannot survive without adhesion on a substrate [9]. The surface properties of substrates play an important role in the cell adhesion. The substrate properties to affect the cell adhesion are the hydrophilicity, charge, stiffness, and roughness [10-12]. To investigate the effect of substrate surface on the cell behavior of adhesion, proliferation, and differentiation, materials with different surface properties have been used [13]. On the other hand, the surface property has been changed chemically or physically by various methods, such as the graft polymerization, oxidation, coating, and roughening [10]. Acrylamide, acrylic acid, and 2-(dimethylamino) ethyl methacrylate have been used for the graft polymerization [14, 15]. Treatment of PET with NaOH aqueous solution increases carboxyl and hydroxyl groups on the surface due to the chemical breakage of PET main chain [16]. As one trial to increase functional groups onto the substrate surface, a SAM technique of alkanethiols has been used [17]. Alkanethiols are self-assembled on a gold-modified surface by the binding of thiolate

groups to the gold molecules and the van der Waal's interaction between the methylene groups of alkanethiols neighboring to each other. The surface topography has been changed by the sand blast and etching [12]. In addition to the physicochemical modification of substrate surface, the surface has been biologically modified. In general, cells adhere on the ECM through the biological interaction between the ECM component and the corresponding integrin receptor on the cell membrane [18, 19]. The integrin receptor interacts with a tripeptide motif, RGD, which is naturally sequenced in the ECM molecule, such as fibronection, vitronectin, collagen, and laminin [18]. When modified with the coating and immobilization of ECM molecule or RGD, the surface can be modified to biologically interact with the integrin receptor of cells [20-24]. Growth factor is another biological component which may affect the cell-substrate interaction. Generally, growth factors have been added into the medium of cell culture to activate the functions of cells and their proliferation and differentiation [25]. It is recognized that the biological effect of growth factors on cells is similar to that of the ECM interacting with the corresponding integrin [19]. Growth factor is also a substance which affects the cell behavior when coated on the substrate surface. There are four action modes of growth factors to bind to the receptor, such as juxtacrine, autocrine, paracrine, and endocrine fashions [26]. Among them is the juxtacrine fashion where a growth factor anchored on the plasma membrane of cells or extracellular matrix acts on the cell receptor. Based on the mechanism of juxtacrine fashion, the surface modification by coating and immobilization of growth factors is expected to affect the cell behavior [27-29]. Although these have been reported to evaluate the effect of substrate surface on the adhesion, proliferation, and differentiation of cells [6-8], little has been performed for fat-derived stem cells, except for the chondrogenic differentiation on an alginate gel [30].

Generally, cell culture has been being extensively performed on a two-dimensional substrate in a static condition. Considering in vivo conditions, cells naturally adhere on the three-dimensional substrate of ECM. It is no doubt that the three-dimensional culture gives cell better conditions for their proliferation and differentiation than the culture on the two-dimensional substrate [31, 32]. Three-dimensional cells culture is influenced by the bulk properties of three-dimensional substrates, including the porosity, pore size, and thickness of sponges and non-woven fabrics or the fiber diameter of fabrics [33-35]. The bulk properties of substrates can be controlled by the preparation procedure of substrates, such as the solution-casting, salt-leaching, and glass transition [33-36]. For the three-dimensional cell culture, the culture method also affects the cell behavior. For example, by the static culture method, cells proliferate non-uniformly inside of the substrate and the number of cells proliferated is higher around the substrate surface than that inside the substrate [37]. Various culture methods have been tired to tackle the problem of non-uniform proliferation [38]. For example, the culture medium is agitated, stirred, or flowed for

efficient cell adhesion on the substrate and the subsequent proliferation and differentiation [38-43]. However, no researches have been reported on the culture of fat-derived stem cells for the efficient proliferation and differentiation by different culture methods. In addition, fat-derived stem cells have hardly been cultured on three-dimensional substrates by different culture methods.

To investigate the interaction between cells and the substrate, the cell adhesion, proliferation, and differentiation have been evaluated only from the viewpoint of the number and morphology of cells, and their protein or polysaccharide production [13, 44-46]. However, it is possible that interaction of cells with the substrate gives a stimulus to the surface receptor to affect the intracellular signaling pathways. The medium movement induced by the agitated and stirred culture method also gives a stimulus to cells [47]. It has been demonstrated that the intercellular signal pathway is activated by both the chemical and mechanical signals through integrins to the activation of FAK [9, 18, 19]. It is recognized that the FAK activation induces the cell spreading through the organization of actin fibers [9]. The activation of FAK subsequently induces the activation of ERK [19]. The ERK activation relates to the survival, proliferation, and differentiation of cells [19, 48]. Therefore, to investigate the cell-substrate interaction in more detail, the cell behavior on different substrates and culture methods should be evaluated in terms of the activation of intercellular signals.

The objective of this thesis is to obtain fundamental information on two- and three-dimensional substrates to culture human fat-derived stem cells. In Part I, fat-derived stem cells were cultured on two-dimensional substrates with different surface properties. Various substrates with different water wettabilities or those surface modified by the SAM, bFGF-coating, and RGD-immobilization techniques were used to evaluate the effect of surface property on the cell behavior. In Part II, fat-derived stem cells were cultured on three-dimensional substrates with different surface properties by various culture methods. The PET non-woven fabrics of three-dimensional substrate were prepared from the fiber with different diameters and the surface was changed by NaOH treatment and RGD immobilization to evaluate the behavior of fat-derived stem cells cultured on the substrates.

Chapter 1 describes the proliferation and osteogenic and adipogenic differentiation of human fat-derived stem cells cultured on two-dimensional substrates with different surface properties. The substrates used were polymer films with different water wettabilities, glass or a cell culture dish, and the dish coated with collagen type I or IV, gelatin, and bFGF. When compared among the polymer substrates without protein coating and glass, the number of cell proliferated became maximum for substrates with surface water contact angles of 60-80°. Osteogenic differentiation was indentical, irrespective of the substrates type, whereas adipogenic differentiation was different between the substrate types.

According to the results of Chapter 1 that the proliferation and adipogenic differentiation of human fat-derived stem cells depended on the surface property of substrates, the surface of substrates chemically modified by the SAM technique was used for further investigation. The SAM technique can easily modify the gold surface of substrates with various types of functional groups. Mixture of two types of functional groups controls the surface properties, such as hydorphilicity by mixture of OH and CH_3 groups and charge by mixture of OH and COOH or OH and NH_2 groups. In Chapter 2, human fat-derived stem cells were cultured on two-dimensional substrates modified with the SAM to investigate the proliferation and differentiation profiles of cells. The substrates used had one or two types of different functional groups (OH, CH_3, NH_2, and COOH groups) on the surface. The cell spreading area and the proliferation level were higher for the SAM of NH_2 or COOH or the mixed SAM of OH and NH_2 or OH and COOH groups than those of other substrates. For the SAM of NH_2 or COOH groups, the strong activation of ERK1/2 was observed, while the mRNA expression of connective tissue growth factor (CTGF) and cystein-rich 61 (CYR61) was enhanced. The proliferation of the cells was significantly suppressed by adding an inhibitor of ERK1/2. The mRNA expression of PPARγ2 was significantly induced by adding the inhibitor. It is concluded that the proliferation and adipogenic differentiation of adipo-stromal cells depended on the chemical composition of substrates surface, although the extent was influenced by that of ERK1/2 activation.

Since bFGF coated on the cell culture dish inhibited adipogenic differentiation in Chapter 1, further investigation of bFGF was performed from the viewpoint of addition modes of bFGF. In Chapter 3, human fat-derived stem cells were cultured with bFGF in the solution and coated forms to investigate their adipogenic differentiation. The morphology and number of cells were similar for both the bFGF modes. However, the ERK1/2 activation of cells incubated with bFGF in the medium was stronger and lasted for a longer time period than that of bFGF in the coated form. When adipo-stromall cells were incubated in an adipogenic differentiation medium, irrespective of the mode of bFGF added, the mRNA expression of PPRAγ2 and aP2, the GPDH activity, and the intracellular accumulation of oil lipids were all suppressed. bFGF in the solution form inhibited the PPARγ2 expression to a high extent compared with that in the coated form. It is possible that bFGF-induced ERK1/2 activation consequently inhibited the adipogenic differentiation of adipo-stromal cells.

Chapter 4 describes the adhesion of human fat-derived stem cells cultured on two-dimensional substrates modified with RGD peptide. Proteins used for substrate coating in Chapter 1 have the RGD peptide sequence in the molecules and affected cell behavior. The different densities and gradients of carboxyl groups on the surface for RGD immobilization were prepared by an exchanged SAM technique which is similar to that in Chapter 2. The densities and

gradients were controlled by the immersion time in ethanol solution containing other alkanethiols with carboxyl groups. RGD was immobilized to achieve an immobilization density of 3.0-12 pmol/cm^2 (2.3-8.9 ng/cm^2) on the SAM. The SAM technique was easy and reproducible to modify the surface of cell substrate by the RGD immobilization. The spreading and survival rate of cells increased with an increase in the density of RGD immobilized on the SAM, although no dependence of the RGD density on the number of cells adhered was observed. This finding suggests the possible that the cell adhesion is regulated by controlling the RGD density or gradient of cell substrates.

Chapter 5 describes the proliferation and osteogenic differentiation of human fat-derived stem cells on PET non-woven fabrics by different culture methods. The PET non-woven fabrics were three-dimensional substrates prepared from PET fibers with various diameters and with different porosities. The largest number of cells initially adhered was observed in the non-woven fabrics prepared from the PET fiber with a diameter of 22.0 μm, irrespective of the fabric porosity. The cell proliferation became better in order of the stirred (spinner flask) > the agitated > the static culture methods. The static culture method tended to enable cells to enhance the osteogenic differentiation, in contrast to the stirred and agitated culture methods. It is concluded that the fabric fiber diameter and the culture method greatly affected the proliferation and differentiation of cells in the non-woven fabrics.

Chapter 6 describes the adhesion and proliferation of human fat-derived stem cells for two- and three-dimensional PET substrates modified with RGD peptide. The PET films and non-woven fabrics were used as the two- and three-dimensional substrates, respectively. Since in Chapters 2 and 3, the introduction of COOH groups and the RGD immobilization enhanced the cell adhesion, the two PET substrates were similarly treated with NaOH aqueous solution to generate carboxyl groups on the surface, and then RGD was immobilized on the substrates surface through the groups. Cells were seeded and cultured on the PET substrates by the static and agitated culture methods. The PET film of two-dimensional substrate by the static culture method was more effective in the cell adhesion than the PET non-woven fabrics of three-dimensional one while the phosphorylation of FAK became higher for PET film. There was no difference in the cell proliferation between the two- and three-dimensional substrates by the static culture method. The surface modification by NaOH treatment and RGD immobilization increased the cell adhesion for the PET non-woven fabrics. The cell behavior was experimentally supported in terms of the FAK phosphorylation profile. The cell proliferation was enhanced by the agitated culture for the PET non-woven fabrics, comparing with that by the static method. The cell proliferation of three-dimensional substrates was enhanced by the agitated culture, whereas it was not influenced by the surface property of substrates, but by the culture methods.

In summary, the surface modification with the SAM technique was useful for two-dimensional substrates to enhance the adhesion, proliferation, and differentiation of human fat-derived stem cells. Comparing the adhesion and proliferation of human fat-derived stem cells between the two- and three-dimensional substrates, the advantageous importance as the culture substrate depended on the culture conditions. For the three-dimensional substrate, the cell behavior was influenced by the culture method to a great extent compared with the surface property.

REFERRENCES

1. J. G. Toma, M. Akhavan, K. J. Fernandes, F. Barnabe-Heider, A. Sadikot, D. R. Kaplan and F. D. Miller *Nat Cell Biol* **3**, 778 (2001)

2. J. Y. Lee, Z. Qu-Petersen, B. Cao, S. Kimura, R. Jankowski, J. Cummins, A. Usas, C. Gates, P. Robbins, A. Wernig and J. Huard *J Cell Biol* **150**, 1085 (2000)

3. K. Kawate, H. Yajima, H. Ohgushi, N. Kotobuki, K. Sugimoto, T. Ohmura, Y. Kobata, K. Shigematsu, K. Kawamura, K. Tamai and Y. Takakura *Artif Organs* **30**, 960 (2006)

4. M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig and D. R. Marshak *Science* **284**, 143 (1999)

5. G. Ferrari, G. Cusella-De Angelis, M. Coletta, E. Paolucci, A. Stornaiuolo, G. Cossu and F. Mavilio *Science* **279**, 1528 (1998)

6. P. A. Zuk, M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim and M. H. Hedrick *Mol Biol Cell* **13**, 4279 (2002)

7. P. A. Zuk, M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz and M. H. Hedrick *Tissue Eng* **7**, 211 (2001)

8. J. M. Gimble and F. Guilak *Curr Top Dev Biol* **58**, 137 (2003)

9. P. J. Reddig and R. L. Juliano *Cancer Metastasis Rev* **24**, 425 (2005)

10. Y. Ikada *Biomaterials* **15**, 725 (1994)

11. D. Karamichos, R. A. Brown and V. Mudera *J Biomed Mater Res A* **83**, 887 (2007)

12. D. W. Hamilton and D. M. Brunette *Biomaterials* **28**, 1806 (2007)

13. Y. Tamada

14. A. Kishida, H. Iwata, Y. Tamada and Y. Ikada *Biomaterials* **12**, 786 (1991)

15. T. Richey, H. Iwata, H. Oowaki, E. Uchida, S. Matsuda and Y. Ikada *Biomaterials* **21**, 1057 (2000)

16. Y. Li, T. Ma, S. T. Yang and D. A. Kniss *Biomaterials* **22**, 609 (2001)

17. A. Ulman *Chem Rev* **96**, 1533 (1996)

18. U. Hersel, C. Dahmen and H. Kessler *Biomaterials* **24**, 4385 (2003)

19. W. Guo and F. G. Giancotti *Nat Rev Mol Cell Biol* **5**, 816 (2004)

20. D. L. Hern and J. A. Hubbell *J Biomed Mater Res* **39**, 266 (1998)

21. K. H. Park, K. Na and H. M. Chung *Biotechnol Lett* **27**, 227 (2005)

22. G. Maheshwari, G. Brown, D. A. Lauffenburger, A. Wells and L. G. Griffith *J Cell Sci* **113** **(Pt 10)**, 1677 (2000)

23. M. Schuler, G. R. Owen, D. W. Hamilton, M. de Wild, M. Textor, D. M. Brunette and S. G. Tosatti *Biomaterials* **27**, 4003 (2006)

24. J. J. Yoon, S. H. Song, D. S. Lee and T. G. Park *Biomaterials* **25**, 5613 (2004)

25.　S. Tsutsumi, A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi and Y. Kato *Biochem Biophys Res Commun* **288**, 413 (2001)

26.　A. B. Singh and R. C. Harris *Cell Signal* **17**, 1183 (2005)

27.　G. Chen, Y. Ito and Y. Imanishi *Biochim Biophys Acta* **1358**, 200 (1997)

28.　Y. Naka, A. Kitazawa, Y. Akaishi and N. Shimizu *J Biosci Bioeng* **98**, 348 (2004)

29.　Y. Ito, G. Chen and Y. Imanishi *Biotechnol Prog* **12**, 700 (1996)

30.　H. A. Awad, M. Q. Wickham, H. A. Leddy, J. M. Gimble and F. Guilak *Biomaterials* **25**, 3211 (2004)

31.　S. Scaglione, A. Braccini, D. Wendt, C. Jaquiery, F. Beltrame, R. Quarto and I. Martin *Biotechnol Bioeng* **93**, 181 (2006)

32.　B. Inanc, A. E. Elcin and Y. M. Elcin *Tissue Eng* **12**, 257 (2006)

33.　Y. S. Moon, H. Uyama, S. Inoue and Y. Tabata *Chemistry Letters* **35**, 564 (2006)

34.　S. L. Ishaug-Riley, G. M. Crane-Kruger, M. J. Yaszemski and A. G. Mikos *Biomaterials* **19**, 1405 (1998)

35.　T. Ren, J. Ren, X. Jia and K. Pan *J Biomed Mater Res A* **74**, 562 (2005)

36.　A. Hokugo, Y. Kubo, Y. Takahashi, A. Fukuda, K. Horiuchi, K. Mushimoto, S. Morita and Y. Tabata *Tissue Eng* **10**, 978 (2004)

37.　D. Wendt, A. Marsano, M. Jakob, M. Heberer and I. Martin *Biotechnol Bioeng* **84**, 205 (2003)

38.　J. M. Kelm and M. Fussenegger *Trends Biotechnol* **22**, 195 (2004)

39.　Y. Takahashi and Y. Tabata *Tissue Eng* **9**, 931 (2003)

40.　Y. Takahashi and Y. Tabata *J Biomater Sci Polym Ed* **15**, 41 (2004)

41.　H. Okubo, M. Matsushita, H. Kamachi, T. Kawai, M. Takahashi, T. Fujimoto, K. Nishikawa and S. Todo *Artif Organs* **26**, 497 (2002)

42.　V. I. Khaoustov, G. J. Darlington, H. E. Soriano, B. Krishnan, D. Risin, N. R. Pellis and B. Yoffe *In Vitro Cell Dev Biol Anim* **35**, 501 (1999)

43.　C. Bassleer, P. Gysen, J. M. Foidart, R. Bassleer and P. Franchimont *In Vitro Cell Dev Biol* **22**, 113 (1986)

44.　M. Balcells and E. R. Edelman *J Cell Physiol* **191**, 155 (2002)

45.　N. Faucheux, R. Schweiss, K. Lutzow, C. Werner and T. Groth *Biomaterials* **25**, 2721 (2004)

46.　B. G. Keselowsky, D. M. Collard and A. J. Garcia *J Biomed Mater Res A* **66**, 247 (2003)

47.　M. E. Gomes, H. L. Holtorf, R. L. Reis and A. G. Mikos *Tissue Eng* **12**, 801 (2006)

48.　F. Bost, M. Aouadi, L. Caron and B. Binetruy *Biochimie* **87**, 51 (2005)

PART I

CULTURE OF HUMAN FAT-DERIVED STEM CELLS ON TWO-DIMENSIONAL SUBSTRATES WITH DIFFERENT SURFACE PROPERTIES

Chapter 1

Proliferation and differentiation of human fat-derived stem cells on substrates with different water contact angles

INTRODUCTION

It is no doubt that stem cells are important for tissue engineering which is one of the biomedical technologies to induce tissue regeneration at a body defect. Several recent studies have demonstrated that the cells of high proliferation and differentiation potentials can be isolated from various adult tissues [1, 2]. Among them, it is reported that stem cells derived from fat tissues differentiate not only into adipogenic cells, but also into osteogenic, chondrogenic, myogenic, and neurogneic cells, similarly to the MSC isolated from the bone marrow [3-8]. MSC have been proven to be multipotent [9, 10]. However, for MSC applications to tissue regeneration, there are some problems to be resolved, including the pain of patients for isolation, the difficulty in isolation, and low number of cells present in the bone marrow. The fat-derived stem cells are advantageous over the MSC for the problematic points. Generally, the volume of fat tissues isolatable is larger than that of bone marrow in a patient body. It will be easier and less painful to collect the fat-derived stem cells from patients. However, for the clinical application of fat-derived stem cells, it is preferable to allow cells to effectively proliferate without losing their differentiation potential from the viewpoint of decrease in the collection volume.

Recently it has been reported that bFGF has an activity to accelerate the proliferation of human MSC [11]. Moreover, the incubation with the growth factor enabled MSC to maintain osteogenic, chondrogenic, and adipogenic potentials *in vitro*. However, no research has been done to evaluate the effect of bFGF on the differentiation potential of fat-derived stem cells isolated from human fat tissue. *In vivo* formation of fat tissue has been experimentally confirmed by using human fat-derived stem cells proliferated in the presence of bFGF [12].

Scaffold is defined as the matrix which provides cells an environment suitable to their proliferation and differentiation. Various scaffolds for MSC have been studied, for example hydroxyapatite, poly(lactic-co-glycolic acid) or collagen for osteogenic differentiation, alginate for chondrogenic differentiation, and collagen for myogenic differentiation and tendon repair [13-18]. As scaffolds for fat-derived stem cells, basement membrane extract (Matrigel) and collagen have been used to induce formation of fat tissue *in vivo* [19-21]. However, little scaffold research has been performed for fat-derived stem cells of multilineage differentiation potential, except that

alginate, agarose, and gelatin gels are used as a scaffold to induce chondrogenic differentiation of fat-derived stem cells [8].

This study was undertaken to obtain fundamental information about the nature of human fat-derived stem cells from the viewpoint of their proliferation and differentiation properties. Fat-derived stem cells were proliferated in the presence or absence of bFGF, and their proliferation behavior and differentiation induction into adipogenic and osteogenic cells were evaluated. Several polymer films with different water wettabilities, glass, a culture plate or that coated with collagen type I or IV, gelatin, and bFGF was used as the culture substrate. The cells proliferated in different bFGF concentrations were cultured on the substrates to investigate the effect of the substrate type on the proliferation and differentiation of fat-derived stem cells.

EXPERIMENTAL

Characterization of substrates

Table 1 summaries various substrates used to culture fat-derived stem cells. All the polymer films were kindly supplied from various private companies and washed with methanol in an ultrasound bath (BRANSON 2510, Yamato Co., Ltd., Tokyo, Japan), followed by rising with DDW to exclude oily substrates from the film surface. Every polymer film was punched out into round films of 14 mm in diameter. The films and glass slips (14 mm in diameter) were sterilized by their immersion into 70 vol% ethanol aqueous solution for overnight and then rinsed twice with autoclaved PBS for the following cell culture experiment. To prepare substrates coated with different proteins, 0.01 wt% of collagen type I or type IV (kindly supplied by Niita Gelatin Co., Osaka, Japan) aqueous solution in HCl, 0.01 wt% of aqueous gelatin solution, and 20 μg/ml of bFGF solution in PBS (200 μl) were poured into each well of 24-well multiwell culture plates (#3626, Corning Co., New York), followed by leaving for 1 hr at room temperature. The gelatin (type A from Porcine skin, #40K0920, Sigma Co., Missouri) solution was sterilized by milipore filtration, while other solutions of original sterilization were used. The protein-coated culture plates were rinsed twice with PBS. The culture plate without protein coating and glass were used as control substrates.

The water wettability of polymer films and protein-coated culture plates was measured to assess their surface hydrophobicity [22]. A water drop (9 μL) was dropped onto the surface of films, glass, and culture plates, and the water contact angle was measured at 25 ^{o}C on a geometer (Contact angle meter CA-X, Kyowa Interface Science Co., Ltd., Saitama, Japan) 0, 30, and 60 sec later. The measurement was done independently 15 times for each sample.

Table 1. The abbreviation of culture substrates used

Code	Full name	Abbreviation	Contact angle (degree)
1	Collagen type IV-coated culture plate	collagen typeIV	16.2±1.4
2	bFGF-coated culture plate	bFGF	16.5±1.2
3	Gelatin-coated culture plate	gelatin	20.4±1.7
4	Collagen type I-coated culture plate	collagen typeI	24.0±1.6
5	Glass	glass	48.5±1.9
6	Poly(vinyl alcohol)	PVA	50.2±2.2
7	24-well multiwell culture plate	culture plate	63.9±4.5
8	Nylon 6,6	Nylon	69.7±1.1
9	Poly(ethylene terephthalate)	PET	75.5±0.7
10	Polypropylene	PP	91.7±1.6
11	Polyethylene	PE	100.9±0.5
12	Silicone	Si	106.4±2.2
13	Poly(tetrafluoroethylene-co-hexafluoropropylene)	6F	109.9±0.4
14	Polytetrafluoroethylene	Teflon	117.7±1.5

Isolation and culture of human fat-derived stem cells with bFGF

Fat-derived stem cells were primarily isolated from the human fat tissue that was collected in the reduction mammaplastic surgery of breast cancer patients after the informed consent was obtained at Kyoto University Hospital. The fat tissue (5 ml) was washed with PBS to carefully remove blood cells, then minced, and digested by 520 U/ml collagenase (Nitta Gelatin Co.) at 37 ^{o}C for 15 min under shaking [12, 19]. The digested was suspended in Medium 199 (Sigma Co.) containing 10 vol% fetal calf serum (FCS, #AJL12371, Hyclone, Utah) and 20 μM ethylenediamineteraacetic acid (EDTA). The suspension was filtered through a 200-μm nylon mesh and centrifuged at 1,000 rpm for 5 min at 4 ^{o}C. After washing twice with the Medium 199 containing 10 vol% FCS (control medium), the cells obtained were suspended in the control medium and cultured at 37 ^{o}C in a 75-cm^2 culture flask (430720, Corning Inc., New York). Non-adherent cells were removed 1 day later and adherent cells were cultured in the control medium containing bFGF concentrations of 0, 1, 10, 100, 1,000, and 10,000 ng/ml. The passage number of cells in this step was defined as 'passage 1 (P 1)'. After incubation for 10 to 14 days, the cells reached near confluence. The cells proliferated were detached with 0.25% trypsin solution containing 7 μM EDTA in PBS, and used for the following studies as the cells of P 2 or further subcultured one to four times at a density of 3×10^4 cells/cm^2.

Proliferation and oteogenic and adipogenic differentiation

The fat-derived stem cells proliferated in bFGF concentrations of 0, 1, 10, 100, 1,000, and 10,000 ng/ml (P 1) were placed into each well of culture plates at a density of 1×10^4 cells/cm^2. The cells of P 2 were further proliferated in the control medium (500 μl/well) containing bFGF

concentrations of 0, 1, 10, 100, 1,000, and 10,000 ng/ml for 1-9 days. To evaluate the effect of the cell passage number on the proliferation of cells, the cells of P 1-5 proliferated in the bFGF concentration of 1,000 ng/ml were placed into each well of culture plates at a density of 1×10^4 cells/cm^2. To evaluate the effect of several substrates on the proliferation of cells, the cells proliferated in 1,000 ng/ml of bFGF (P 1) were placed into each well of culture plates on the bottom which the polymer films and glass slip had been set or that of culture plates coated with various proteins (1×10^4 cells/cm^2). The medium used (500 μl/well) was the control medium containing 1,000 ng/ml of bFGF.

The fat-derived stem cells proliferated in different concentrations of bFGF (P 1) were placed into each well of culture plates at a density of 3×10^4 cells/cm^2. The cells of P 2 were incubated in the control medium (500 μl/well) containing 28 μM L-ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone (osteogenic medium) for 28 days [23]. To evaluate effect of the cell passage number and cell initial density on the osteogenic differentation of cells, the cells proliferated in 1 ng/ml of bFGF (P 1-3) were placed into each well of culture plates at densities of 1×10^4 and 3×10^4 cells/cm^2 and incubated in the osteogenic medium (500 μl/well) for 28 days. To evaluate the effect of several substrates on the cell osteogenic differentiation, the cells proliferated in 1 ng/ml of bFGF (P 1) were placed on the substrates (3×10^4 cells/cm^2) and cultured in the osteogenic medium for 7 days.

The fat-derived stem cells proliferated in different concentrations of bFGF (P 1) were placed into each well of culture plates at a density of 1×10^4 cells/cm^2. The cells of P 2 were further proliferated in the control medium (500 μl/well) containing different concentrations of bFGF for 1-9 days. For adipogenic differentiation, the cells were incubated in DME/Ham's F12 medium (500 μl/well) containing 0.05 μM insulin, 0.2 nM 3,5,3'-triiodothyronine, 100 nM transferrin, 17 μM calcium pantotenate, 33 μM biotin, and 100 nM dexamethasone (ITT medium) for 30 days [24]. To evaluate the effect of the cell initial density on the adipogenic differentiation, the cells proliferated in 1,000 ng/ml of bFGF (P 1-4) were placed into each well of culture plates at different densities of 1×10^3, 3×10^3, 1×10^4, 3×10^4, and 1×10^5 cells/cm^2 and proliferated in the control medium (500 μl/well) containing 1,000 ng/ml of bFGF for 5 days. Then, the cells were incubated in the ITT medium for 30 days. To evaluate the effect of several substrates on the adipogenic differentiation of cells, the cells proliferated in 1,000 ng/ml of bFGF (P 1) were placed on various substrates 1×10^4 cells/cm^2 and further proliferated in the control medium containing 1,000 ng/ml of bFGF for 5 days. Then, the cells were incubated in the ITT medium for 30 days.

Observation of cell morphology and cell staining

The morphology of fat-derived stem cells was observed by a phase-contrast microscopy

(IX70, OLYMPAS OPICAL Co., Ltd., Tokyo, Japan). The cells were stained with von Kossa and oil red O to histologically evaluate the osteogenesis and adipogenesis, respectively. The cells incubated in the osteogenic medium were rinsed twice with PBS and fixed with 10% formalin solution in PBS for 10 min at room temperature. After washing with DDW, the cells were incubated with 5 w/v% silver nitrate solution under bright light for 2 hr, and then incubated with 5 w/v% sodium thiosulfate solution for 3 min. The cells incubated in the ITT medium were rinsed twice with PBS and fixed with 10% formalin solution in PBS for 1 hr at 4 ^{o}C. The cells were rinsed twice with PBS and 250 μl of 60% isopropanol in DDW and stained with 250 μl of the oil red O solution per well for 30 min at room temperature. An oil red O (0.3 wt%) stock solution in isopropanol (6 ml) was mixed with 4 ml of DDW and the mixed solution was left for 10 min at room temperature, followed by filtration through a 0.22-μm filter to prepare the oil red O solution. The wells were rinsed with 250 μl of 60% isopropanol in DDW. The picture of stained cells was randomly taken (5 pictures/well) to assess the area ratio of oil red O-stained cells to total cells by a computer program of Image-Pro (Planetron, Inc., Tokyo, Japan).

Measurement of cell number

The number of cells proliferated was measured by a DNA assay method [25]. The fat-derived stem cells were rinsed twice with PBS, underwent a freeze and thaw process, and finally incubated in 1 ml of aqueous solution containing 0.2 mg/ml sodium dodecyl sulfate (SDS), 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate for 1 hr at 37 ^{o}C for cell lysis. The cell lysate (100 μl) was mixed 100 μl solution of 1 μl/ml Hoechst 33258 dye solution (bisbenzimide H33258 fluorochrome trihydrochloride DMSO solution, #L2E4809, Nacalai Tesque, Inc.,Kyoto, Japan), 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate, and then the fluorescence intensity of mixed solution was measured on a fluorescence spectrophotometer (Spectra Max Gemini Em, Molecular Device Japan Co., Osaka, Japan) at exciting and emission wavelengths of 355 and 460 nm. The cell number was determined by use of calibration curve prepared from the fluorescent measurement for the cells of known number.

Measurement of ALP activity, calcium content, osteocalin content

The ALP activity was assayed by a commercial kit (alkaline phosphor B-test wako, #TQ717, Wako Pure Chemical Industries, Ltd., Osaka, Japan) based on the absorbance measurement of a p-nitrophenol product generated by ALP digestion [23]. Briefly, 80 μl of cell lysate prepared by the method described above was mixed with 100 μl of 6.7 mM p-nitrophenyl phosphate aqueous solution, followed by leaving at 37 ^{o}C for 15 min. After mixing 1 ml of 20 mM NaOH aqueous solution, the absorbance of the solution mixture was measured at 405 nm by a

spectorophotometer (Versa Max, Molecular Device Japan Co.) to assess the ALP activity.

Cells were incubated in 1 ml of 0.5 N HCl solution by shaking for 4 hr at 4 $^{\circ}$C. The calcium amount of HCl solution obtained was determined by a commercial kit (Calcium C-test wako, #TH994, Wako Pure Chemical Industries, Ltd., Osaka, Japan) [23]. Briefly, 10 μl of the cell lysate was mixed with 1 ml of 0.88 M monoethanolamine aqueous solution. After mixing 100 μl of 69 mM 8-hydroxxyquinoline and 0.63 mM o-cresolphthalein complexon aqueous solution, the solution mixture was allowed to stand for 5 min. The absorbance of the solution mixture was measured at 570 nm by the spectorometer (Versa Max, Molecular Device Japan Co.) to assess the calcium amount.

The osteocalcin content was assayed by the conventional ELISA in a 10-mm petridish for cells incubated in 15 ml of the control medium or osteogenic medium at a density of 3×10^4 cells/cm^2. Before the osteocalcin assay, the cells were incubated in the serum-free control medium (4 ml) for 24 hr [6, 26]. The osteocalcin in the serum-free medium was measured by a commercial kit (Gla-Type Ostecalcin EIA kit, Takara Bio Inc., Shiga, Japan).

Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity

GPDH activity was assayed by a commercial kit (GPDH activity measurement kit, JFL003, Sangi Co., Ltd., Tokyo, Japan) based on the absorbance measurement of a NADH reduction [24]. Cells were rinsed twice with PBS and homogenized in a buffer solution of the kit using a handy sonic (UR-20, Tomy Seiko Co., Ltd., Tokyo, Japan) on ice. After mixing a reaction solution of kit, the absorbance of the solution mixture was measured at 340 nm by the spectorophotometer (Versa Max, Molecular Device Japan Co.) to assess the GPDH activity.

Statistical analysis

All the data were statistically analyzed by Post hocs Fisher's PLSD and expressed as the mean ± the standard deviation of the mean. Statistical significance was accepted at p<0.05.

RESULTS

Proliferation of cells

Figure 1 shows the phase-contrast micrographs of fat-derived stem cells proliferated for 9 days in different concentrations of bFGF. Cells incubated in the absence of bFGF flatly expanded, while those incubated in the presence of bFGF also expanded although the extent was smaller and the frame of cells became clear with an increase in the bFGF concentration. Figure 2 gives the time

profile of cell proliferation in different concentrations of bFGF. The cell proliferation depended on the bFGF concentration. The cells proliferated in bFGF concentrations of 1,000 and 10,000 ng/ml at significantly higher rates than those in the absence of bFGF or the lower bFGF concentrations.
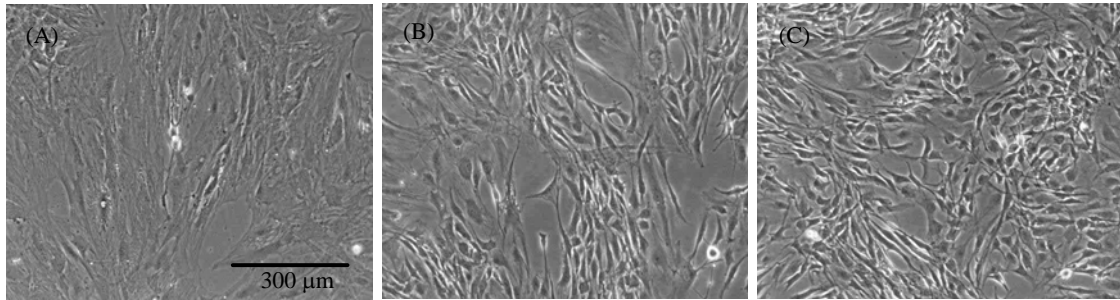
Figure 1. Phase-contrast microscopic photographs of fat-derived stem cells proliferated on the culture plate in the control medium containing bFGF concentrations of 0 (A), 10 (B), and 1,000 ng/ml (C) for 9 days. The cell density of cells seeded initially was 1×10^4 cells/cm^2.

Figure 2. Proliferation profiles of fat-derived stem cells on the culture plate in the control medium containing different concentrations of bFGF for 1 (○), 5 (△), 9 days (□). The cell density of cells seeded initially was 1×10^4 cells/cm^2.
*, $p<0.05$: significant against the number of cells proliferated in the control medium containing bFGF concentrations of 0, 1, 10, and 100 ng/ml at the corresponding time period of incubation.

Figure 3 shows the effect of the cell passage number on the proliferation of cells in the control medium containing 1,000 ng/ml of bFGF for 1-9 days. The cell proliferation tended to become slow with the increasing passage number and was not observed at the P 6.
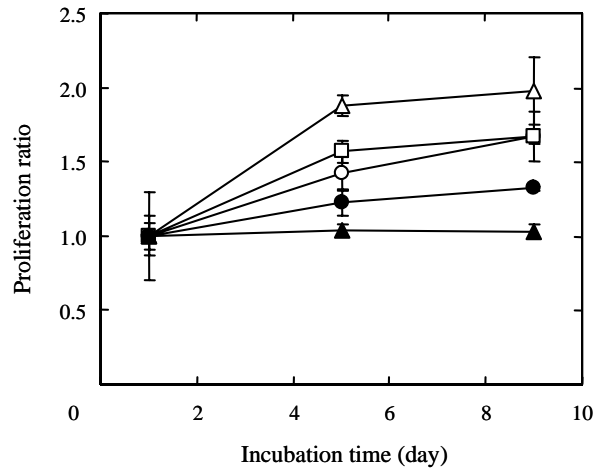
Figure 3. Effect of the cell passage number on the proliferation of fat-derived stem cells in the control medium containing a bFGF concentration of 1,000 ng/ml. The passage number was 2 (○), 3 (△), 4 (□), 5 (●) or 6 (▲). The cell density of cells seeded initially was 1×10^4 cells/cm^2. The Y-axis indicates the number ratio of cells to those proliferated for 1 day at the corresponding passage number.

Osteogenic and adipogenic differentiation of cells

Figure 4 shows the photographs of von Kossa-stained fat-derived stem cells proliferated in different concentrations of bFGF and incubated in the osteogenic medium. There was not big difference in the mineral deposition between the cells proliferated in the different concentrations of bFGF. Figure 5 shows the ALP activity, calcium content, and osteocalcin content of cells proliferated in different concentrations of bFGF. For incubation time periods of 7, 14, 21, and 28 days, the ALP activity level of cells proliferated in the presence of bFGF significantly was higher than that in the absence of bFGF. The time profile of ALP activity depended on the bFGF concentration. The ALP activity of cells proliferated in 1 ng/ml of bFGF increased fastest within the initial 7 days to reach a maximum at 14 days, and thereafter decreased. The ALP activity of cells proliferated in higher bFGF concentrations tended to maintain for longer time periods. The calcium content of cells proliferated in 1,000 and 10,000 ng/ml of bFGF was significantly lower than that in the absence of bFGF. However, the calcium content increased to reach the same level of cells proliferated in the absence of bFGF. An increase in the osteocalcin content of cells cultured in the osteogenic medium was observed, whereas cultured in the control medium was not effective.
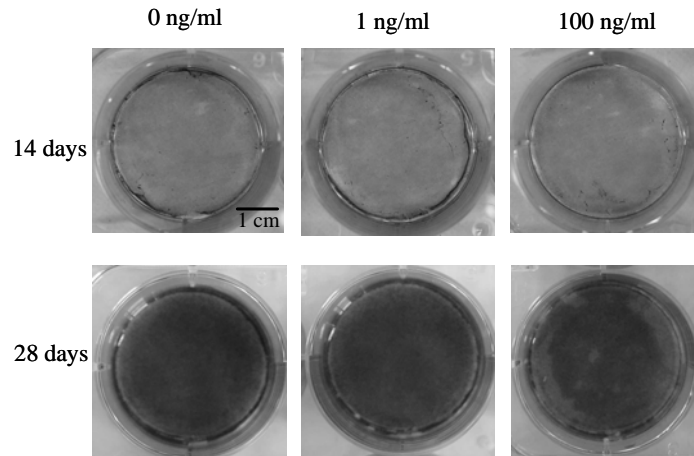
Figure 4. Photographs of von Kossa-stained fat-derived stem cells proliferated in the control medium containing bFGF concentrations of 0, 1, and 100 ng/ml. The cell density of cells seeded initially was 3×10^4 cells/cm^2. The cells were proliferated in different bFGF concentrations and incubated in the osteogenic medium further for 14 and 28 days.
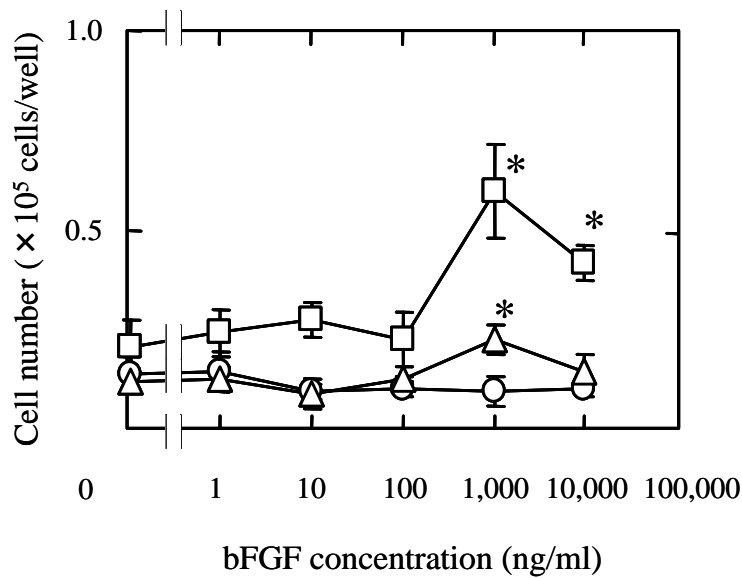

Figure 5. ALP activity (A) and Calcium content (B) of fat-derived stem cells proliferated in the control medium containing different bFGF concentrations. The cells were proliferated in the control medium containing bFGF concentrations of 0, 1, 10, 100, 1,000, and 10,000 ng/ml and incubated in the osteogenic medium further for 7 (□), 14 (▦), 21 (■) ,and 28 days (▨). The cell density of cells seeded initially was 3×10^4 cells/cm^2. (C) Osteocalcin content of fat-derived stem cells proliferated in the control medium containing bFGF concentration of 1 ng/ml and incubated in the osteogenic medium or control medium further for 28 days.

*, $p < 0.05$: significant against the ALP activity of fat-derived stem cells proliferated in the absence of bFGF at the corresponding time period of incubation.

†, $p < 0.05$: significant against the calcium content of fat-derived stem cells proliferated in the absence of bFGF at the corresponding time period of incubation.

‡, $P < 0.05$: significant against the osteocalcin content of fat-derived stem cells incubated in the control medium.

Figure 6 shows the effect of the cell passage number and initial cell density on the osteogenic differentiation of cells. For both the ALP activity and calcium content, the values of P 2 and P 3 cells were significantly high compared with those of P 4 cells. The ALP activity of cells at the higher density seemed to be higher than that at the lower density. The time profile of ALP activity for P 2 cells was different between the initial cell densities.

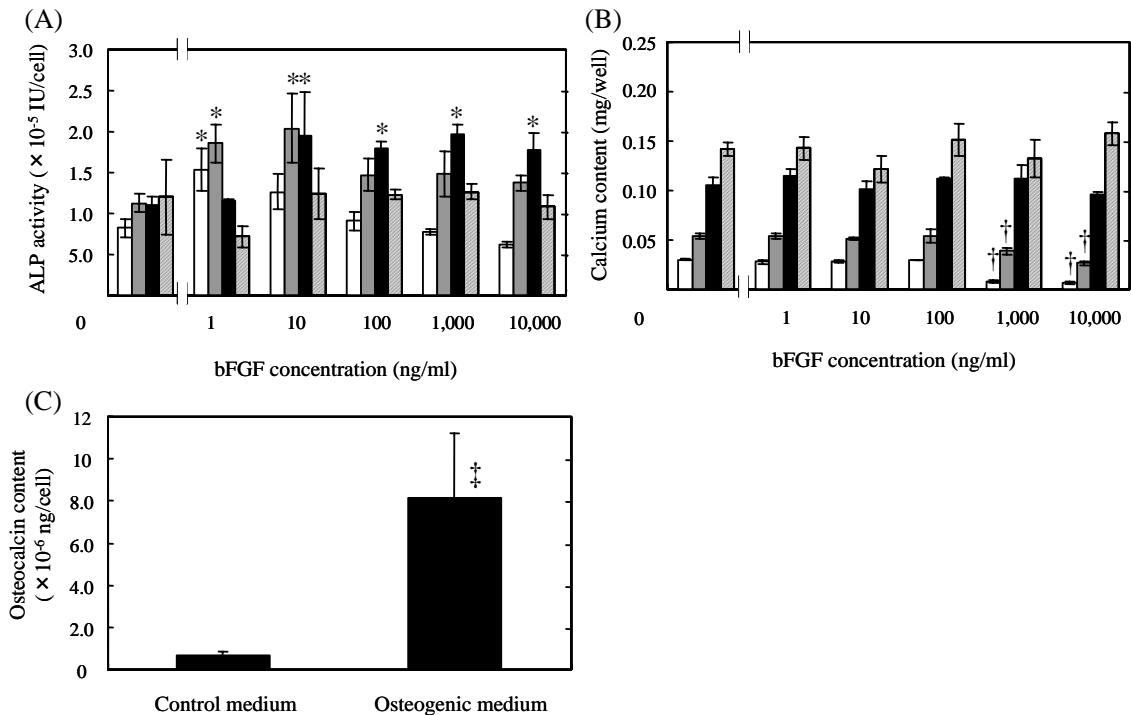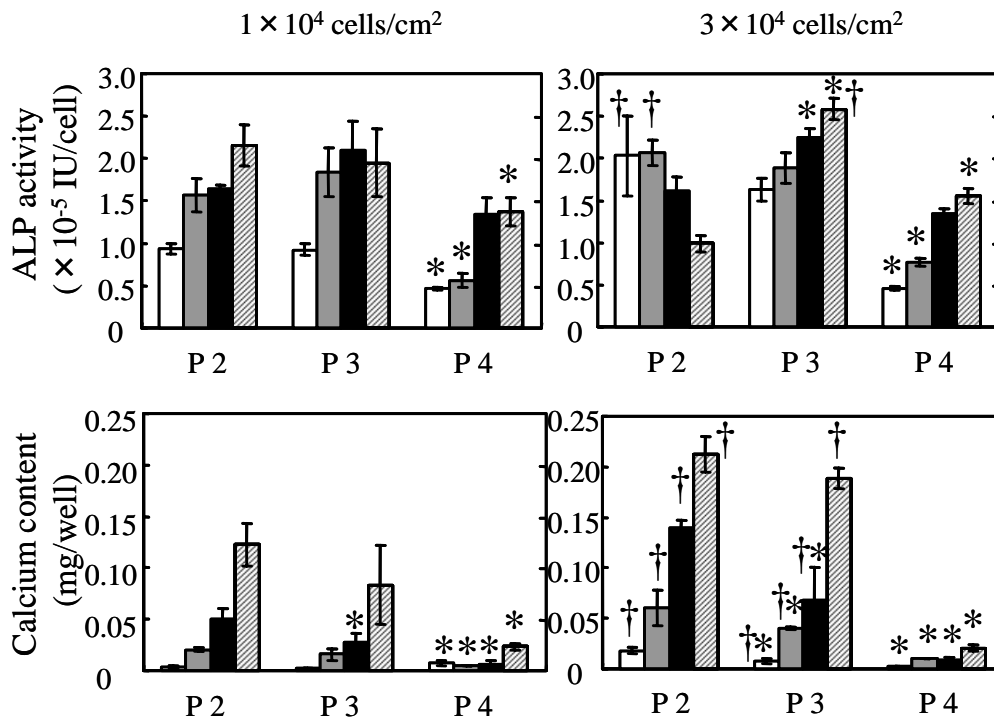Figure 6. Effect of the cell passage number and initial cell density on the ALP activity and calcium content of fat-derived stem cells proliferated in the control medium containing a bFGF concentration of 1 ng/ml. Then, the cells were incubated in the osteogenic medium further for 7 (□), 14 (▨), 21 (■), and 28 days (▨).The cell density of cells seeded initially was 1×10^4 or 3×10^4 cells/cm^2.
*, p<0.05: significant against the ALP activity or calcium content of P 2 fat-derived stem cells at the corresponding time period of incubation and cell density.
†, p<0.05: significant against the ALP activity or calcium content of fat-derived stem cells seeded initially at 1×10^4 cells/cm^2 at the corresponding time period of incubation and passage number.

Figure 7 shows phase-contrast microscopic photographs of oil red O-stained fat-derived stem cells proliferated in bFGF concentrations of 0 and 1,000 ng/ml on the culture plate and incubated in the ITT medium further for 30 days. The cell density of cells seeded initially was 1×10^4 and 3×10^4 cells/cm^2, and incubated in 1 μg/ml of bFGF for 5 days. There was difference in the oil red O-stained area between the cells. Few oil red O-stained areas were observed for the cells proliferated in 0 ng/ml of bFGF, while adipogenic differentiation was observed for the cells

proliferated in 1,000 ng/ml of bFGF. Figure 8 shows the effect of bFGF concentrations on the adipogenic differentiation of cells proliferated for different time periods. When proliferated in bFGF concentrations higher than 10 ng/ml, the cells showed significant increase in the oil red O-stained area by further incubation in the ITT medium. The area depended on the time period for cell proliferation before the adipogenesis culture. In bFGF concentrations of 100, 1,000, and 10,000 ng/ml, the oil red O-stained area significantly increased in the case of cells proliferated for 3 days or longer which is in contrast to the bFGF concentrations of 0, 1, and 10 ng/ml. In 10,000 ng/ml bFGF, the oil red O-stained area increased with the incubation time of cell proliferation. A significant increase was observed only for 10 days of adipogenesis incubation, becoming higher than incubation time period (Figure 8 (C)). The similar dependence of GPDH activity on the bFGF concentration was observed. When cultured in bFGF concentrations higher than 10 ng/ml, a significant increase in the GPDH activity was observed, while the oil red O-stained area increased in a time-dependent manner. Figure 9 shows the effect of the cell passage number and initial cell density on the adipogenic differentiation of fat-derived stem cells. When the cell density was higher 1×10^5 cells/cm^2, the oil red O-stained area was significantly large compared with the lower cell densities. The adipogenesis was detected at cell densities of 3×10^3 cells/cm^2 or larger, although no influence of the passage number was observed.
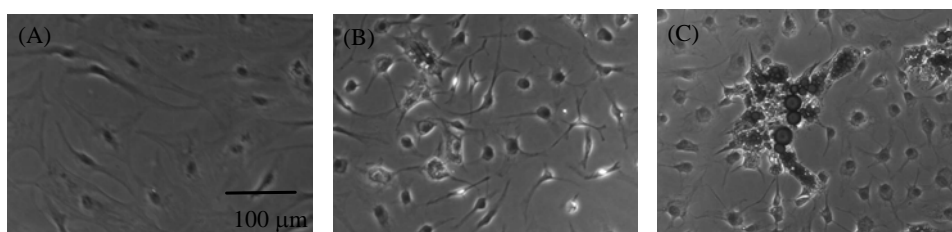
Figure 7. Phase-contrast microscopic photographs of oil red O-stained fat-derived stem cells proliferated in the control medium containing bFGF concentrations of 0 (A) and 1,000 ng/ml (B and C) on culture plate and incubated in the ITT medium further 30 days. The cell density of cells seeded initially was 1×10^4 (A and B) or 3×10^4 cells/cm^2 (C).
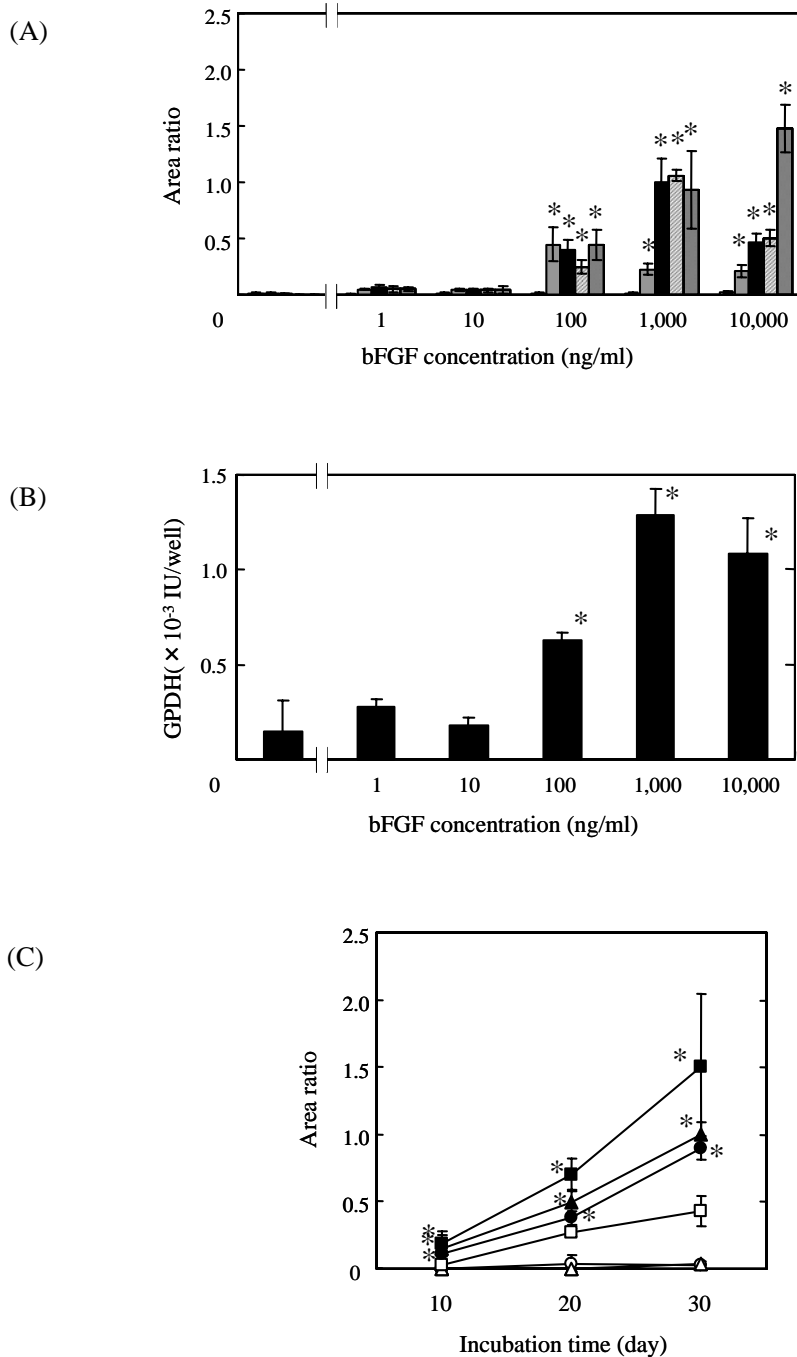
Figure 8. Effect of the bFGF concentration on the oil red O-stained area of fat-derived stem cells. The cells were proliferated in the control medium containing bFGF concentrations of 0, 1, 10, 100, 1,000, and 10,000 ng/mL for 1 (□), 3 (▨), 5 (■), 7(▨), and 9 days (▨), and incubated in the ITT medium further for 30 days (A). Effect of the bFGF concentration of the GPDH activity of fat-derived stem cells proliferated in the control medium containing different bFGF concentrations for 5 days and incubated in the ITT medium further for 30 days (B). The time dependence of oil red O-stained area. The cells were cultured in the control medium containing different bFGF concentrations of 0 (○), 1 (△), 10 (□), 100 (●), 1,000 (▲), and 10,000 ng/ml (■) for 5 days and incubated in the ITT medium for 10-30 days (C). These cell densities of cells seeded initially were 1×10^4 cells/cm^2.
*, p<0.05: significant against the adipogenic differentiation of fat-derived stem cells proliferated in the control medium containing bFGF concentrations of 0, 1, and 10 ng/ml of bFGF.
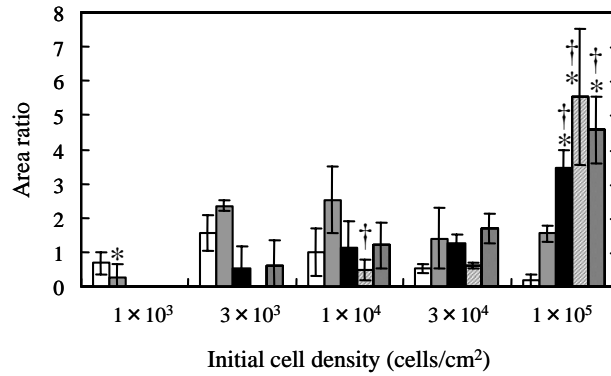
Figure 9. Effect of the cell passage number and initial cell density on the oil red O-stained area of fat-derived stem cells proliferated in the control medium containing a bFGF concentration of 1,000 ng/ml for 5 days and incubated in the ITT medium further for 30 days. The passage number was 2 (□), 3 (▨), 4 (■), 5 (▨).The cell density of cells seeded initially was 1×10^3, 3×10^3, 1×10^4, 3×10^4, or 1×10^5 cells/cm^2.

*, p<0.05: significant against the adipogenic differentiation of fat-derived stem cells seeded initially at 1×10^4 cell/cm^2 at corresponding passage number.

†, p<0.05: significant against the adiopigenic differentiation of fat-derived stem cells (P 2) at the corresponding cell density

Proliferation of cells on substrates with different water contact angles

Figure 10 shows the effect of culture substrates on the proliferation of fat-derived stem cells for various time periods. When compared among the polymer substrates without protein coating and glass, the cell number became maximum for substrates with the water contact angles of 60-80 ^{o}. The large cell number was observed for the culture plates coated with gelatin, collagen type I, collagen type IV, and bFGF, irrespective of the protein type. The similar dependence of substrate contact angle was observed for cell proliferated for 5 and 9 days although the higher cell proliferation was observed on the glass and culture plate (Figure 10). The culture plates coated with various proteins were better substrates to enhance cell proliferation.
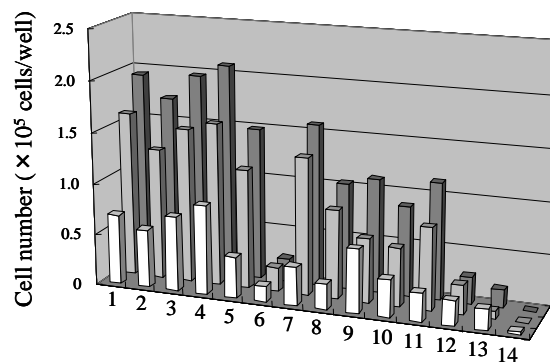
Fisure 10. Proliferation of fat-derived stem cells on various substrates with different contact angles for 1 (□), 5 (▨), 9 days (■) in the control medium containing FGF-2 concentration of 1,000 ng/mL. The cell density of cells seeded initially was 1×10^4 cells/cm^2. See the substrate number in Table 1.

Osteogenic and adipogenic differentiation of cells on substrates with different water contact angles

Figure 11 shows the effect of several substrates on the osteogenic differentiation of fat-derived stem cells. The ALP activity of cells increased with an increase in the cell number.

Figure 12 shows the adipogenic differentiation of fat-derived stem cells incubated on different substrates. The oil red O-stained area was larger for the culture plates coated with gelatin, collagen type I, and collagen type IV than that of other substrates. No difference in the adipogenic differentiation was observed between culture substrates.
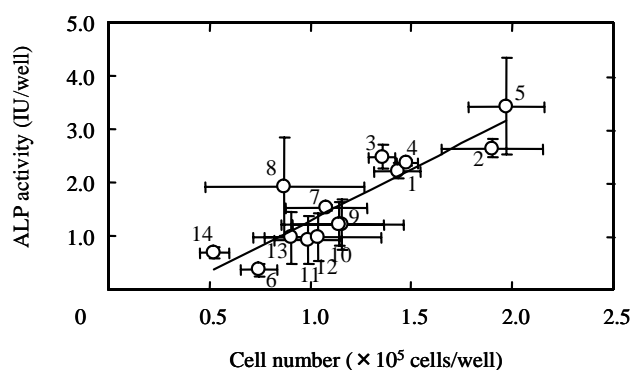
Figure 11. ALP activity of fat-derived stem cells cultured on various substrates with different contact angles as a function of cell number. The cells were proliferated in the control medium containing a bFGF concentration of 1 ng/ml and incubated in the osteogenic medium further for 7 days to evaluate their ALP activity. The cell density of cells seeded initially was 3×10^4 cells/cm^2. See the substrate number in Table 1.
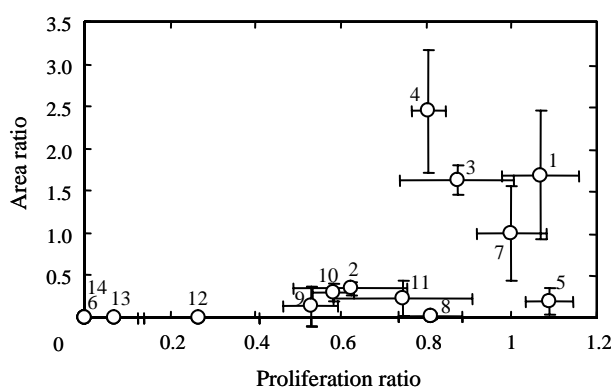
Figure 12. Oil red O-stained area of fat-derived stem cells cultured on various substrates with different contact angles as a function of cell number ratio. The cells were proliferated in the control medium containing a bFGF concentration of 1,000 ng/ml for 5 days and incubated in the ITT medium further for 30 days. The cell density of cells seeded initially was 1×10^4 cells/cm^2. The Y-axis indicates the number ratio of cells after culture in 1,000 ng/ml of bFGF for 5 days to those on culture plate. See the substrate number in Table 1.

DISCUSSION

The fat-derived stem cells isolated from human fat tissue have been investigated in some groups [3-8]. Hedrick et al. have demonstrated that fat-derived stem cells are stable over long-time culture, maintaining a consistent population doubling rate and exhibiting low level of senescence. In this study, fat-derive stem cells slowly proliferated in the absence of bFGF. Although the addition of bFGF enabled cells to proliferate faster, the cell proliferation became slow with the increasing passage number. They used the cells which were isolated from subcutaneous fat tissues obtained from an elective liposuction procedure, whereas the cells used here were isolated from visceral fat tissues. The different proliferation potential of cells may be due to the difference in the tissue type for cell isolation.

There are some data on the effect of bFGF concentration on the proliferation and differentiation potential of human MSC into osteogenic, adipogenic, and chondrogenic cells. The presence of 1 ng/ml bFGF accelerated the MSC proliferation and maintained their differentiation [11]. After expanding with bFGF (5 ng/ml) and dexamethasone, MSC displayed a sharper increase in the ALP activity and the activity reached higher level than that of control medium-expanded cells in osteogenic medium [27]. Incubation of MSC in the osteogenic medium containing bFGF (2.5 ng/ml) increased the number and size of the colonies that formed, and the number of cells recovered in STOP-1 and ALP (osteoprogenitor) fraction [28]. However, the dependence of the fat-derived stem cells behavior on the bFGF concentration was different. This may be explained in terms of cell difference. Incubation of fat-derived stem cells in the adipogenic medium containing bFGF (0.1-100 ng/ml) had no effect on their proliferation and morphology [29]. The high concentration effect of bFGF on the behavior of fat-derived stem cells has not been studied.

The effect of passage number on the differentiation potential of cells has been examined in several other studies. The osteogenic potential of MSC decreased with an increase in the passage number [30, 31]. The similar phenomenon was also observed for fat-derived stem cells. The cells, when proliferated in the presence of bFGF, lost the ability of differentiation into osteogenic cells with the increasing passage number (Figure 6). On the other hand, the adipogenic potential tended to depend on the passage number although the dependence was different by the density of cells seeded initially. It is reported that the ostoegenic potential of MSC is influenced by the initial cell density [32]. For the osteogenic differentiation of MSC, the calcium content depended on the initial density (3×10^3 and 5×10^3 cells/cm^2), while the ALP activity did not. Our data showed the similar dependency of cell density. The extent of cell adipogenic differentiation was also different between cells initially seeded at different densities.

Adipogenesis was evaluated by various methods, for example oil red O or Nile red

staining, the RT-PCR assay of PPAR-γ and aP2, and measurement of GPDH activity [4, 24]. For the oil red O staining, adipogenesis was quantitated by measuring the area of cells stained with oil red O. The dependence of bFGF concentration on the adipogenic differentiation based on the staining method was similar to that of GPDH activity. This indicates that the oil red O-staining method was easy and accurate to evaluate cell adipogenesis.

For the cell scaffold for tissue regeneration, it is preferable that three-dimensional scaffolds are used to investigate the effect of scaffold material property on the cell behavior. However, there are many factors to be considered for the scaffolds, such as porosity, pore size, the pore interconnectivity, surface property, topography, and chemical composition. Such a factor dispersity often causes difficulty in the scaffold research. Therefore, in this study, two-dimensional substrates were used to investigate only the influence of substrate surface property on the proliferation and differentiation of fat-derived stem cells. The proliferation and differentiation profiles of fat-derived stem cells depended on the type of substrates and the bFGF concentration for cell proliferation. Higher cell proliferation was observed for the substrates with water contact angles of 60-80° as well as the protein-coated culture plates. Tamada et al. demonstrated that the initial adhesion of mouse fibroblasts and the subsequent proliferation were greatly influenced by the water contact angle of substrates [33]. The similar influence was observed for rat MSC.

It is well recognized that interaction between cells and the ECM greatly affects on their proliferation and differentiation. It has been shown that similar ALP activity (IU/cell) was observed for MSC proliferated on the culture plate, irrespective of the collagen type I coating, whereas the MSC proliferation on the collagen-coated culture plate was higher than those on the culture plate [34]. The result was also observed for the present experiment. There was no influence of substrate type on the ALP activity per cells. On the other hand, the oil red O-staind area of fat-derived stem cells on the collagen type I, collagen type IV, and gelatin-coated culture plates was larger than that on the culture plate. It has been reported that collagen type V or VI plays an active role in adipogenic differentiation [35]. Contrary to the osteogenic differentiation, adipogenic differentiation of fat-derived stem cells may be influenced not only by adipogenic medium, but also the surrounding property of cells.

REFERENCES

1.   J. G. Toma, M. Akhavan, K. J. Fernandes, F. Barnabe-Heider, A. Sadikot, D. R. Kaplan and F. D. Miller *Nat Cell Biol* **3**, 778 (2001)

2.   J. Y. Lee, Z. Qu-Petersen, B. Cao, S. Kimura, R. Jankowski, J. Cummins, A. Usas, C. Gates, P. Robbins, A. Wernig and J. Huard *J Cell Biol* **150**, 1085 (2000)

3.   P. A. Zuk, M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz and M. H. Hedrick *Tissue Eng* **7**, 211 (2001)

4.   P. A. Zuk, M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim and M. H. Hedrick *Mol Biol Cell* **13**, 4279 (2002)

5.   H. Mizuno, P. A. Zuk, M. Zhu, H. P. Lorenz, P. Benhaim and M. H. Hedrick *Plast Reconstr Surg* **109**, 199 (2002)

6.   Y. D. Halvorsen, D. Franklin, A. L. Bond, D. C. Hitt, C. Auchter, A. L. Boskey, E. P. Paschalis, W. O. Wilkison and J. M. Gimble *Tissue Eng* **7**, 729 (2001)

7.   K. M. Safford, K. C. Hicok, S. D. Safford, Y. D. Halvorsen, W. O. Wilkison, J. M. Gimble and H. E. Rice *Biochem Biophys Res Commun* **294**, 371 (2002)

8.   H. A. Awad, M. Q. Wickham, H. A. Leddy, J. M. Gimble and F. Guilak *Biomaterials* **25**, 3211 (2004)

9.   M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig and D. R. Marshak *Science* **284**, 143 (1999)

10.   G. Ferrari, G. Cusella-De Angelis, M. Coletta, E. Paolucci, A. Stornaiuolo, G. Cossu and F. Mavilio *Science* **279**, 1528 (1998)

11.   S. Tsutsumi, A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi and Y. Kato *Biochem Biophys Res Commun* **288**, 413 (2001)

12.   Y. Kimura, M. Ozeki, T. Inamoto and Y. Tabata *Biomaterials* **24**, 2513 (2003)

13.   T. Yoshikawa, H. Ohgushi and S. Tamai *J Biomed Mater Res* **32**, 481 (1996)

14.   A. S. Goldstein, G. Zhu, G. E. Morris, R. K. Meszlenyi and A. G. Mikos *Tissue Eng* **5**, 421 (1999)

15.   K. Yamanouchi, K. Satomura, Y. Gotoh, E. Kitaoka, S. Tobiume, K. Kume and M. Nagayama *J Bone Miner Res* **16**, 857 (2001)

16.   H. L. Ma, S. C. Hung, S. Y. Lin, Y. L. Chen and W. H. Lo *J Biomed Mater Res A* **64**, 273 (2003)

17.   Y. Hori, T. Nakamura, D. Kimura, K. Kaino, Y. Kurokawa, S. Satomi and Y. Shimizu *J Surg Res* **102**, 156 (2002)

18.   H. A. Awad, D. L. Butler, M. T. Harris, R. E. Ibrahim, Y. Wu, R. G. Young, S. Kadiyala and

G. P. Boivin *J Biomed Mater Res* **51**, 233 (2000)

19.   Y. Kimura, M. Ozeki, T. Inamoto and Y. Tabata *Tissue Eng* **8**, 603 (2002)

20.   D. von Heimburg, S. Zachariah, I. Heschel, H. Kuhling, H. Schoof, B. Hafemann and N. Pallua *Biomaterials* **22**, 429 (2001)

21.   D. von Heimburg, S. Zachariah, A. Low and N. Pallua *Plast Reconstr Surg* **108**, 411 (2001)

22.   M. R. Brunstedt, N. P. Ziats, M. Schubert, S. Stack, V. Rose-Caprara, P. A. Hiltner and J. M. Anderson *J Biomed Mater Res* **27**, 499 (1993)

23.   C. Maniatopoulos, J. Sodek and A. H. Melcher *Cell Tissue Res* **254**, 317 (1988)

24.   H. Hauner, G. Entenmann, M. Wabitsch, D. Gaillard, G. Ailhaud, R. Negrel and E. F. Pfeiffer *J Clin Invest* **84**, 1663 (1989)

25.   J. Rao and W. R. Otto *Anal Biochem* **207**, 186 (1992)

26.   D. Lajeunesse, G. M. Kiebzak, C. Frondoza and B. Sacktor *Bone Miner* **14**, 237 (1991)

27.   O. Frank, M. Heim, M. Jakob, A. Barbero, D. Schafer, I. Bendik, W. Dick, M. Heberer and I. Martin *J Cell Biochem* **85**, 737 (2002)

28.   S. Walsh, C. M. Jefferiss, K. Stewart and J. N. Beresford *Bone* **33**, 80 (2003)

29.   H. Hauner, K. Rohrig and T. Petruschke *Eur J Clin Invest* **25**, 90 (1995)

30.   P. J. Ter Brugge and J. A. Jansen *Tissue Eng* **8**, 321 (2002)

31.   M. J. Coelho, A. T. Cabral and M. H. Fernande *Biomaterials* **21**, 1087 (2000)

32.   C. W. Lan, F. F. Wang and Y. J. Wang *J Biomed Mater Res A* **66**, 38 (2003)

33.   Y. Tamada and Y. Ikada *in: Polymer in Medicine II*, E. Cheiellini, P. Giusti, C. Migliaresi and L. Nicolais (Eds). p.101. (1986)

34.   N. Jaiswal, S. E. Haynesworth, A. I. Caplan and S. P. Bruder *J Cell Biochem* **64**, 295 (1997)

35.   I. Nakajima, S. Muroya, R. Tanabe and K. Chikuni *Differentiation* **70**, 84 (2002)

Chapter 2

Proliferation and adipoigenic differentiation of human fat-derived stem cells on substrates modified with SAM

INTRODUCTION

Fat-derived stem cells which can be isolated from the fat tissue, are capable of differentiating into adpogenic, osteogenic, chondrogenic, neurogenic, and myogenic cells [1-3]. The fat-derived stem cells can be used as a stem cell source for tissue regeneration therapy, similarly to MSC isolated from the bone marrow. To induce cell-based tissue regeneration at a body defect, in addition to stem cells, the scaffolds are often needed since they provide cells an environment suitable to their proliferation and differentiation. Various scaffolds have been studied for the fat-derived stem cells and MSC, for example hydroxyapatite [4], poly(lactic-co-glycolic acid) [5], collagen [6], and alginate [7]. For the material design of scaffold, the surface properties of materials, such as the chemical composition and morphology, will play an important role in the cellular behavior. The effect of scaffold surface property on the proliferation and differentiation of stem cells has been investigated [8, 9]. However, little systematic study about the cellular response in terms of surface chemistry has been performed because of limited type of materials used.

Formation technique of SAM with alkanethiols and silanes has been used for surface modification and the chemical property on surface can be readily modified by SAM compounds with different functional groups [10]. Recently the surface modification of SAM has been performed to design the cell culture substrate, and various kinds of cells, for example fibroblast cell lines [11], epidermoid carcinoma cell lines [12], osteoblast-like cell lines [13-15], osteosarcoma cell lines [16], osteoblast [17], leukocytes [18], epithelial cells [19], neurons [20], and MSC [21], were cultured on the surfaces. It was found from the researches that functional groups on the SAM surface affect the attachment [11, 13, 15, 19], the morphology [11, 14-16], the proliferation [11, 16, 21], the differentiation of cells [15, 16, 21], or the cell cycle [16]. However, no effect of the SAM type on the adipogenic differentiation of cells has been investigated.

This study was undertaken to obtain fundamental information about the proliferation and adipogenic differentiation of human fat-derived stem cells cultured on the substrate of different chemical compositions. Different surfaces were prepared by the SAM technique with several alkanethiols with OH, CH_3, NH_2, and COOH groups or the mixture. It is well known that the balance of non-polar hydrophilic and hydrophobic properties of substrates affects the cell

attachment and proliferation [22]. Thus, in this paper, the OH and CH$_3$ groups were selected to change the surface of cell culture substrates. In addition, cationic and anionic charges also modify the cell behavior. The corresponding electric charges were achieved by combining NH$_2$ and COOH groups with the OH group of hydrophilicity to exclude the hydrophobic influence on the cell response [14]. The surface properties of SAM were characterized by X-ray photoelectron spectroscopy (XPS) [14] and water contact angle measurement [11, 14, 16, 18]. Following the fat-derived stem cells were isolated from human adipose tissue and seeded on the SAM, the attachment, morphology, proliferation, and differentiation of cells were evaluated in terms of the surface chemical composition. We examine the cell behavior in terms of mRNA expression of biosignaling molecules and an intracellular signal activation. The mRNA expression of PPARγ2 and aP2 was a measure to evaluate adipogenic differentiation, while that of connective tissue growth factor (CTGF) and cystein-rich 61 (CYR61) was related to cell proliferation [23].

EXPERIMENTAL

Preparation of substrates modified by a SAM technique

PET film was kindly supplied by Teijin Ltd. (Tokyo, Japan) and washed with methanol in an ultrasound bath (Brason 2510, Yamato Tokyo, Japan), followed by rising with DDW. The PET film was punched out into the round films of 14 mm in diameter, followed by the electrochemical deposition of gold (50 nm thickness) on the film surface (Muranaka Medical Instrument Co., Ltd., Osaka, Japan). The gold-deposited films were immersed for 24 hr in ethanol solution containing 1 mM of 1-undecanethiol (CH$_3$, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), 11-mercapto-1-undecanol (OH, Sigma-Aldrich Co., MO), 11-amino-1-undecanethiol (NH$_2$, Dojindo Laboratories, Co., Kumamoto, Japan), and 11-mercaptoundecanoic acid (COOH, Sigma-Aldrich Co.), or the mixture (OH:X mixing mol ratio=0:100, 25:75, 50:50, 75:25, and 100:0, X=CH$_3$, NH$_2$, and COOH) [14]. The SAM were thoroughly rinsed with ethanol to exclude alkanethiols unreacted and dried.

Characterization of SAM-modified substrates

The surface of SAM was characterized by water contact angle and XPS measurements. The water contact angle was measured with a geometer (contact angle meter CA-X, Kyowa Interface Science, Saitama, Japan) according to the method previously reported [8, 9]. Briefly, a water drop (9 μl) was dropped onto the surface of SAM at room temperature and the water contact angle was measured independently 15 times for each SAM. The XPS data were obtained by using

an Electron Spectroscopy for Chemical Analysis instrument (ESCA-850V, Shimazu, Kyoto, Japan) [14]. The operation conditions for X-ray source were 8 kV and 30 mA below 1×10^{-6} torr. The chemical composition on the SAM surface was calculated by comparing the N or O x-ray photoelectron peak of the mixed SAM with that of mono SAM.

Evaluation of protein adsorption

Absorption of fibronectin and vitronectin onto SAM from fetal calf serum (FCS, #AJL12371, Hyclone Laboratories Inc., UT) was measured by the ELISA method with slight modification [24]. The SAM were placed in Medium 199 (500 µl, Sigma-Aldrich Co.) containing 10 vol% FCS and 1 vol% penicillin-streptomycin (Sigma-Aldrich Co.) at 37 ^{o}C for 1 hr. The SAM were washed with 100 mM of PBS and immersed in 3 wt% skim milk aqueous solution for 1 hr. After washing with PBS containing 0.1 vol% Tween-20 (PBS-T) three times, the films were incubated in Can get signal solution 1 (TOYOBO Co. Ltd., Osaka, Japan) containing anti-fibronectin (1/10,000, LSL Co. Ltd., Shiga, Japan) or anti-vitronectin antibody (1/10,000, Biogenesis Ltd, Poole, UK) for 1 hr. After washing with PBS-T three times, the SAM were incubated in Can get signal solution 2 containing peroxidase-conjugated anti-rabbit IgG antibody (1/2000, Sigma-Aldrich Co.) for 1 hr. Then, they were washed with PBS-T three times and placed in 1.7 mg/ml of o-phenylenediamine in sodium peroxoborate solution (0.75 mg/ml) for 15 min, followed by the addition of 2N H_2SO_4 solution to stop the coloring reaction, The supernatants were collected and transferred to each well of 96-well multiwell plates (#06430135, Greiner Bio-one Co., Ltd, Tokyo, Japan) while the solution absorbance was measured on a microplate reader at 495 nm (Versa max, Molecular Device Japan Co., Osaka, Japan). The absorbance ratio of vitronectin or fibronectin for the SAM sample to the cell culture dish was calculated to evaluate the protein adsorption. For each sample, the measurement was performed two times independently.

Isolation and culture of human fat-derived stem cells

Fat-derived stem cells were primarily isolated from the human fat tissue collected in the reduction mammaplastic surgery of breast cancer patients whose informed consents had been obtained at Kyoto University Hospital. The fat tissue (5 ml) other than cancer and fibrous tissue was washed with PBS to carefully remove blood cells, then minced, and digested by 520 U/ml collagenase (Nitta Gelatin Inc., Osaka, Japan) at 37 ^{o}C for 15 min under shaking [9]. The digested was suspended in Medium 199 containing 10 vol% FCS and 1 vol% penicillin-streptomycin (normal medium). The suspension was filtered through a 200-µm nylon mesh and centrifuged at 1,000 rpm for 5 min at 4 ^{o}C. After washing twice with the normal medium, the cells obtained were suspended in the medium and cultured at 37 ^{o}C in a 75-cm^2 culture flask (430720, Corning, New

York). Non-adherent cells were removed 1 day later and adherent cells were proliferated in the nomal medium containing 1 μg/ml of bFGF kindly supplied from Kaken Pharmaceutical Co., Ltd., Tokyo, Japan [9]. After incubation for 7 to 10 days, the cells density became almost confluent. The cells proliferated were detached with 0.25 wt% trypsin solution containing 7 μM ethylenediaminetetraacetate in PBS and used for the following experiments.

Proliferation and adipogenic differentiation

The SAM was sterilized by immersion in 70 vol% ethanol solution for 1 hr, washed with PBS, and place into each well of cell culture dish (Non-treated microplate, Asahi Techno Glass Co., Tokyo, Japan). Then, fat-derived stem cells suspended in the normal medium (500 μl) containing 1 μg/ml bFGF were placed on each SAM surface at a density of 1×10^4 cells/cm^2. As a control, cells were suspended in FCS-free Medium 199 containing 1 vol% penicillin-streptomycin, 50 nM insulin (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 100 nM transferrin (Wako Pure Chemical Industries Ltd.) and used similarly. Fresh medium was changed every 3 days for cell proliferation. For the inhibition test of ERK1/2, cells were cultured in the normal medium containing 1 μg/ml bFGF as well as 50 μM PD98059 (Calbiochem Inc., Darmstadt, Germany) and placed on each SAM surface for 5 days, and then the cell proliferation was investigated [25]. The morphology of fat-derived stem cells was observed by a phase-contrast microscopy (IX70, Olympus Optical Co., Ltd., Tokyo, Japan) 6 hr after cell culture in the normal medium at a seeding density of 1×10^4 cells/cm^2. The cell spreading area was measured by a computer program of MetaMorph (Molecular Devices Inc., Ontario, Canada). For every SAM surface, the spreading area of 100 cells was measured and averaged.

The fat-derived stem cells were plated onto different SAM at a density of 5×10^4 cells/cm^2 and incubated in the normal medium for first 1 day and then cultured in DME/Ham's F12 medium (500 μl/well) containing 0.05 μM insulin, 0.2 nM 3,5,3'-triiodothyronine, 100 nM transferrin, 17 μM calcium pantotenate, 33 μM biotin, and 100 nM dexamethasone (ITT medium) [26]. Fresh ITT medium was exchanged every 3 days for adipogenic differentiation and the following morphological and biochemical examinations. The morphology of adipogenically differentiated cells was observed similarly after incubation for 30 days in the ITT medium.

Cell staining

Cells were incubated on SAM in the normal medium containing 1 μg/ml bFGF for 6 hr. After rinsing twice with PBS, they were fixed on ice in acetone for 20 min [27]. Cells were washed three times with PBS and the, treated with 3 wt% bovine serum albumin in PBS for 20 min at room temperature. Next, cells were incubated with fluorescein isothiocyanare (FITC)-conjugated

phalloidin (10 μg/ml, Sigma-Aldrich Co.) for 1 hr at room temperature and Hoechst 33258 (1 μg/ml, Invitrogen Co., CA) for 5 min at room temperature to visualize the filamentous actin (F-actin) fiber and nucleus, respectively. Following PBS washing, cells were mounted in FluorSave Reagent (Calbiochem Inc.), to view by a microscope (AxioCam HRc, Carl Zeiss Inc., NY) with an oil immersion objective lens.

Measurement of cell number

The number of cells proliferated was measured by a DNA assay method [28]. The cells were rinsed twice with PBS, underwent the conventional freeze-thaw process, and finally incubated in 1 ml of aqueous solution containing 0.2 mg/ml sodium dodecyl sulfate, 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate for 1 hr to completely lyze cells. The cell lysate (100 μl) was mixed with 100 μl of 1 μl/ml Hoechst 33258 dye solution (bisbenzimide H33258 fluorochrome trihydrochloride dimethyl sulfoxide solution, #L2E4809, Nacalai Tesque, Inc., Kyoto, Japan) containing 9.0 mg/ml NaCl and 4.4 mg/ml sodium citrate. The fluorescence intensity of mixed solution was measured on a fluorescence spectrophotometer (Spectra Max Gemini Em, Molecular Device Japan Co., Osaka, Japan) at exciting and emission wavelengths of 355 and 460 nm. The cell number was determined by the calibration curve prepared from the fluorescent measurement for the known number of cells.

Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity

To evaluate adipogenic differentiation of cells, the GPDH activity was assayed by a commercial kit (GPDH activity measurement kit, JFL003, Sangi Co., Ltd., Tokyo, Japan) [9, 26]. Cells were rinsed twice with PBS and homogenized in a kit buffer solution with a handy sonic (UR-20, Tomy Seiko Co., Ltd., Tokyo, Japan) on ice. After mixing with a kit reaction solution, the absorbance of solution mixture was measured at 340 nm by the spectorophotometer (Versa max) to assess the GPDH activity.

Immunobloting of ERK phosphorylation

The cell lysates prepared were mixed with the Radio-Immunoprecipitation Assay (RIPA) buffer containing protease inhibitors (Sigma-Aldrich Co.).   The protein content of cell lysates was measured using the BioRad Protein Assay Kit (BioRad Laboratories Inc., CA).   The cell lysates were applied to sodium dodecylsulfate polyacrylamide electrophoresis (SDS-PAGE), and transferred to an Immobilon-P membrane (Millipore Co., MA). The membrane was immunoblotted with antibodies against phospho-ERK1/2, ERK1/2 (Cell signaling Technology Inc., MA), and β-actin (Sigma-Aldrich Co.) as an internal control as the diluted ratios of 1/1,000, 1/1,000, and 1/10,000, respectively. After incubation in peroxidase-conjugated anti-mouse or anti-rabbit

secondary antibody (Sigma-Aldrich Co.), the target proteins were immunologically visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Thermo Fisher Scientific Inc., IL).

Real time RT-PCR

RNA was prepared from cells using the RNeasy Plus Mini Kit (Qiagen Co., CA) according to the manufacture's instructions [29]. Reverse transcription reaction was performed with the SuperScript Frist-Stand Synthesis System (Invitrogen Co.), while real time PCR was carried out on the 7500 real time PCR system (Applied Biosystems Japan Ltd., Tokyo, Japan). The reaction was proceeded with about 10 ng cDNA in a total volume of 25 μl containing SYBR Green PCR Master Mix (Applied Biosystems Japan Ltd.) and 10 μM of each primer (Table 1). The reaction mixture was treated at 95 °C for 10 min as the initial process, followed by 40 PCR cycles.   Each cycle consisted of the following three steps; 94 °C for 15 sec, 57 °C for 15 sec, and 72 °C for 1 min. Each mRNA level was normalized to the internal β-actin control.

Table 1. Primers used for real time RT-PCR assays

Name	Primer	
PPARγ2	sense	GAAAGCGATTCCTTCACTGATACA
	antisense	AATAAGGTGGAGATGCAGGCTC
aP2	sense	TACTGGGCCAGGAATTTGAC
	antisense	TGGTTGATTTTCCATCCCAT
CTGF	sense	AAGACACGTTTGGCCCAGA
	antisense	TTGCCCTTCTTAATGTTCTCTTCC
CYR61	sense	GATTGCAGTTGGAAAAGGCA
	antisense	CAGCGTAAGTAAACCTGACTGGTT
β-actin	sense	ATGGTGGGCATGGGTCAGAAGG
	antisense	ACGCACGATTTCCCGCTCGGCC

Data analysis

All the data were statistically analyzed by post-hoc Fisher's PLSD tests and expressed as the mean ± the standard deviation of the mean. Statistical significance was accepted at $p < 0.05$

RESULTS

Characterization of SAM

Figure 1 shows the water contact angle and surface elementary analysis data of SAM prepared with different alkanethiols. The water contact angle of SAM increased with an increase in the molar percentage of CH_3, NH_2, and COOH groups, although the percentage dependence of contact angle was influenced by the group type. The water contact angles on SAM of OH, CH_3, NH_2, and COOH groups were in good accordance with those reported previously [11, 14, 16, 18]. The chemical composition of SAM surface was changed by altering the molar percentage of CH_3, NH_2, and COOH groups used for SAM formation.

(A)   (B)
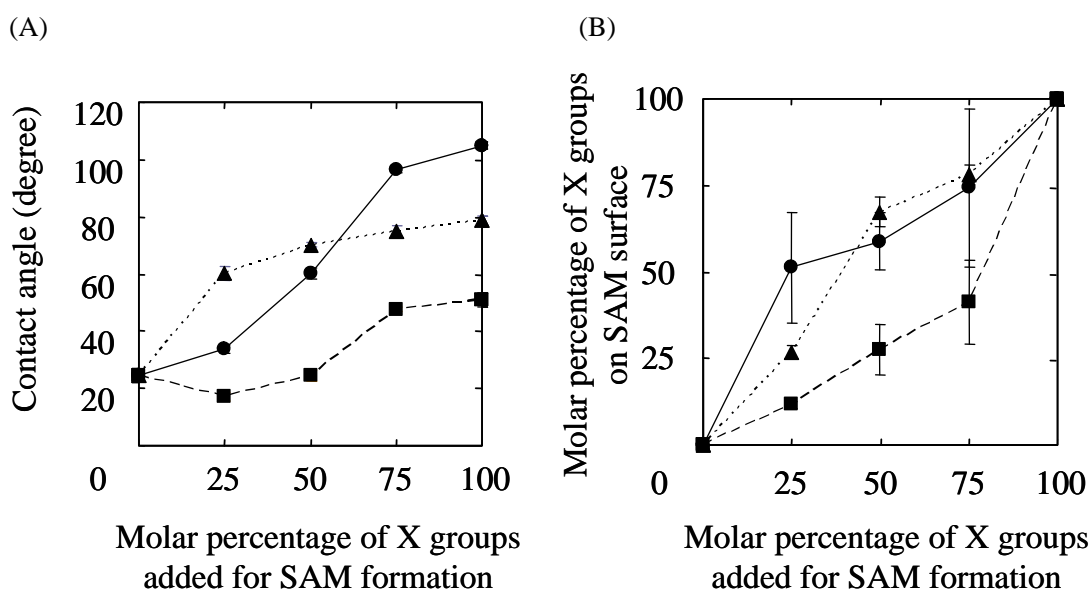
Figure1. Characterization of SAM surfaces by water contact angle (A) and XPS measurements (B). The SAM were prepared by immersing in ethanol solution containing alkanethiols at OH:X mixing ratios of 0:100, 25:75, 50:50, 75:25, and 100:0 : X=CH_3 (●), NH_2 (▲), and COOH groups (■).

Protein adsorption on SAM-modified substrates

Figures 2 (A) and (B) show effect of the SAM composition on the adsorption of vitronectin and fibronectin. The amount of vitronectin adsorbed from FCS increased with an increase of molar percentage of NH_2 group on the mixed SAM of OH and NH_2 groups. No significant difference in the vitronectin adsorption was observed for the mixed SAM of OH and CH_3 or OH and COOH groups. The fibronectin adsorption depended on the molar percentage of the mixed SAM of OH and CH_3 or OH and COOH groups, in contrast to that of the mixed SAM of OH and NH_2 groups.

Figure 2. Adsorption of vitronectin (A) and fibronectin (B) onto the surface of SAM prepared at different OH:X mixing ratio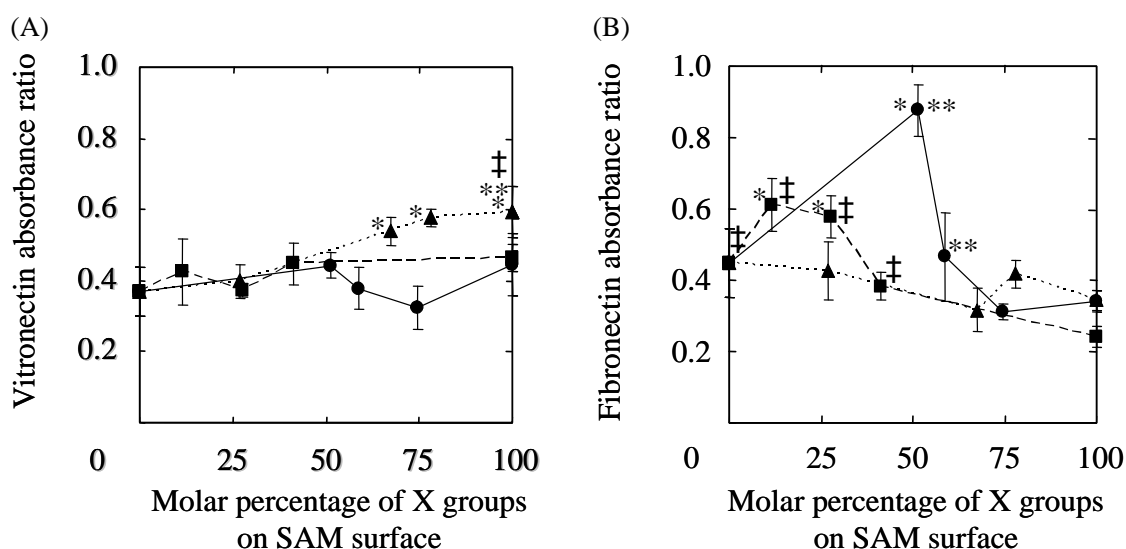s : X= CH_3 (●), NH_2 (▲), and COOH groups (■). The y-axis indicates the ratio of adsorbed protein on SAM to that on cell culture dish. Molar percentage of X on the SAM surface was determined by XPS measurement.

*, $p<0.05$; significant against the absorbance ratio of protein adsorbed onto the SAM of 100% OH group.

**, $p<0.05$; significant against the absorbance ratio of protein adsorbed onto the SAM of 100% CH_3 group.

‡, $p<0.05$; significant against the absorbance ratio of protein adsorbed onto the SAM of 100% COOH group.

Spreading of cells on SAM-modified substrates

Figure 3 (A) shows the F-actin fiber conditions of fat-derived stem cells after 6 hr incubation on SAM of OH, CH_3, NH_2, and COOH groups. For the OH, NH_2, and COOH SAM, actin fibers were observed and cells spread in the present of FCS. On the contrary, cells incubated on the SAM of CH_3 group showed a round shape and the cell morphology was different from that of cells on other materials. When cultured in the absence of FCS, cells were smaller size, irrespective of SAM type. Figure 3 (B) shows the area of cells spread to evaluate the measure of cell morphology. In the presence of FCS, the cell spreading area was larger for the SAM of NH_2, COOH, and the mixed SAM of OH and NH_2 or OH and COOH groups than that on SAM of OH group. The area of cells incubated on the SAM of CH_3 group was smaller even with FCS while smaller area was observed for any SAM sample in FCS-free medium culture. The cell spreading areas on the cell culture dish were 3.0×10^3 and 2.5×10^3 μm^2 for 10 and 0 vol% FCS, respectively.

(A)

(B)
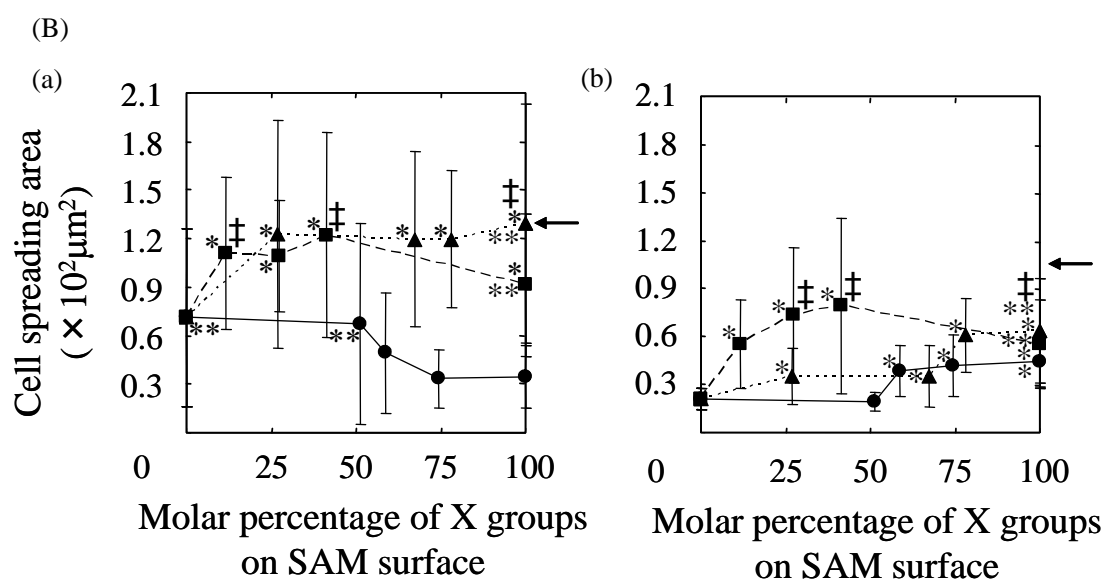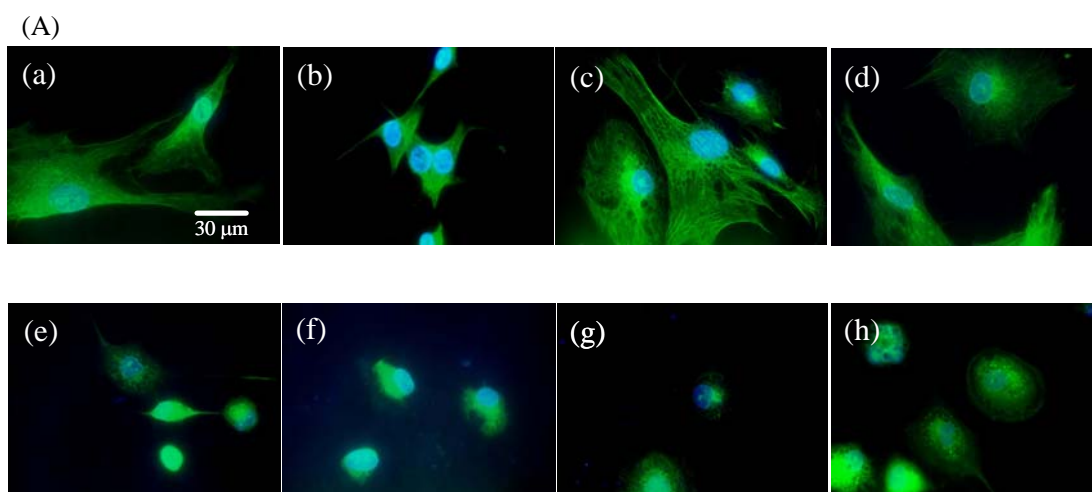
Figure 3. (A) Fluorescent microscopic photographs of fat-derived stem cells incubated for 6 hr in the normal medium with (a-d) or without FCS (e-h) onto the SAM of OH (a and e), CH_3 (b and f), NH_2 (c and g), and COOH groups (d and h).

(B) Spreading areas of fat-derived stem cells incubated for 6 hr in the normal medium with (a) or without FCS (b) onto the SAM prepared at different OH:X mixing ratios : X=CH_3 (●), NH_2 (▲), and COOH groups (■).

Molar percentage of X on the SAM surface was determined by XPS measurement. The arrow indicates the spreading area of fat-derived stem cells onto the cell culture dish.

*, $p<0.05$; significant against the spreading area of cells incubated onto the SAM of 100% OH group.

**, $p<0.05$; significant against the spreading area of cells incubated onto the SAM of 100% CH_3 group.

‡, $p<0.05$; significant against the spreading areas of cells incubated onto the SAM of 100% COOH group.

Proliferation of cells on SAM-modified substrates

Figure 4 (A) shows the phase-contrast micrographs of fat-derived stem cells proliferated on the SAM of OH, CH$_3$, NH$_2$, and COOH groups or the cell culture dish for 1, 5, and 9 days. Cells proliferated on the SAM of NH$_2$ and COOH groups for the first day and showed a spindle shaped morphology, which is similar to those of the cell culture dish. On the contrary, fewer cells were observed on the SAM of OH and CH$_3$ groups and the shape was round and different from that of other samples.
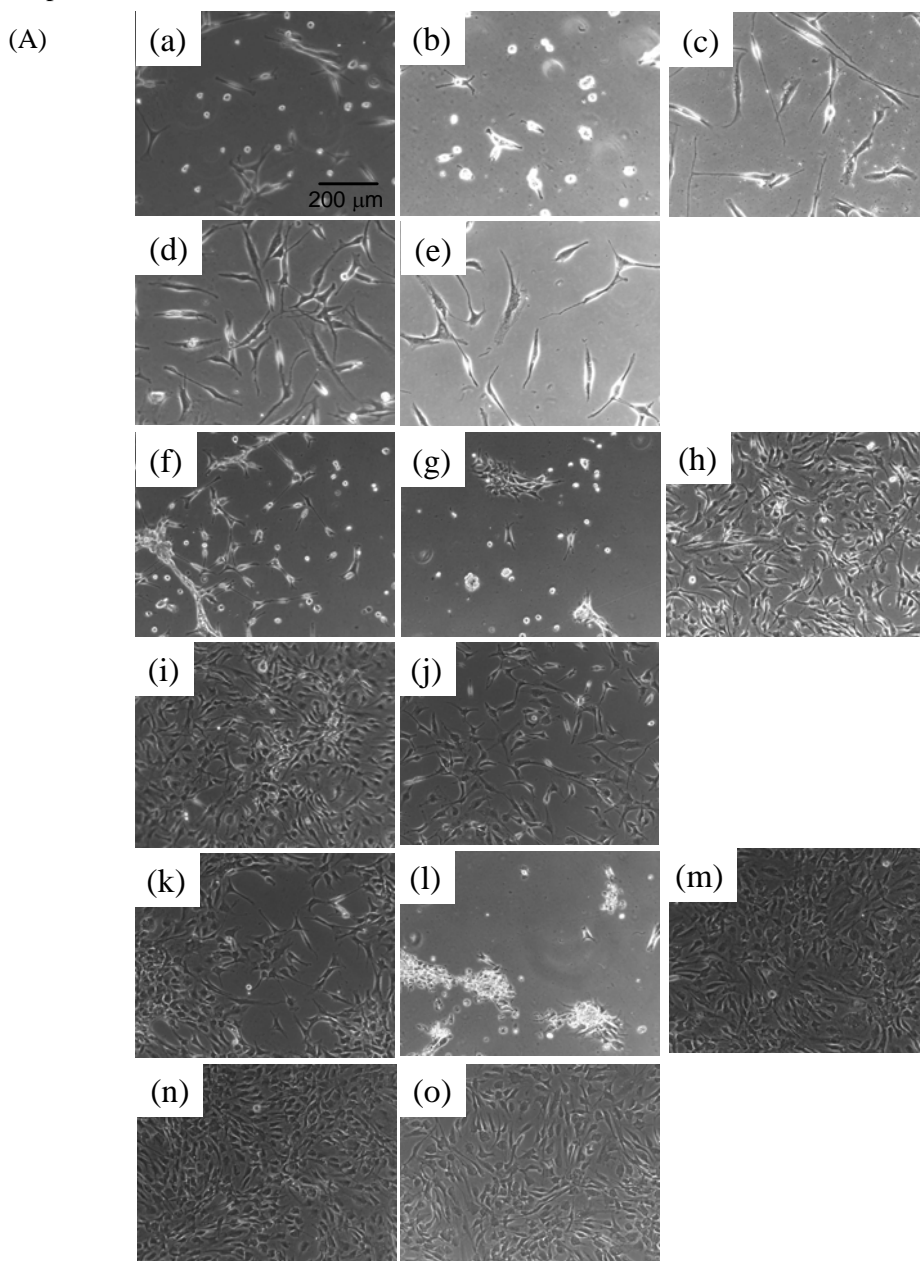
(A)

Figure 4. (A) Phase-contrast microscopic photographs of fat-derived stem cells cultured for 1 (a-e), 5 (f-j), and 9 days (k-o) onto SAM of OH (a, f, and k), CH$_3$ (b, g, and l), NH$_2$ (c, h, and m), and COOH groups (d, i, and n) or cell culture dish (e, j, and o).

Figure 4 (B) shows the cell number on the surface of different SAM. Although the cell number was similar for every SAM surface 1 day after incubation, it greatly depended on the type of SAM 5 and 9 days later. Cells proliferated on the SAM of NH_2, COOH, and the mixed SAM of OH and NH_2 or OH and COOH groups to a significantly high extent compared with those of OH SAM. Mixed SAM of OH and NH_2 or OH and COOH groups even at small percentages significantly increased the cell proliferation on the SAM of OH and CH_3 groups. The cell number on the cell culture dish was 3.6×10^4, 9.9×10^4, and 1.6×10^5 cells/well at 1, 5, and 9 days, respectively.
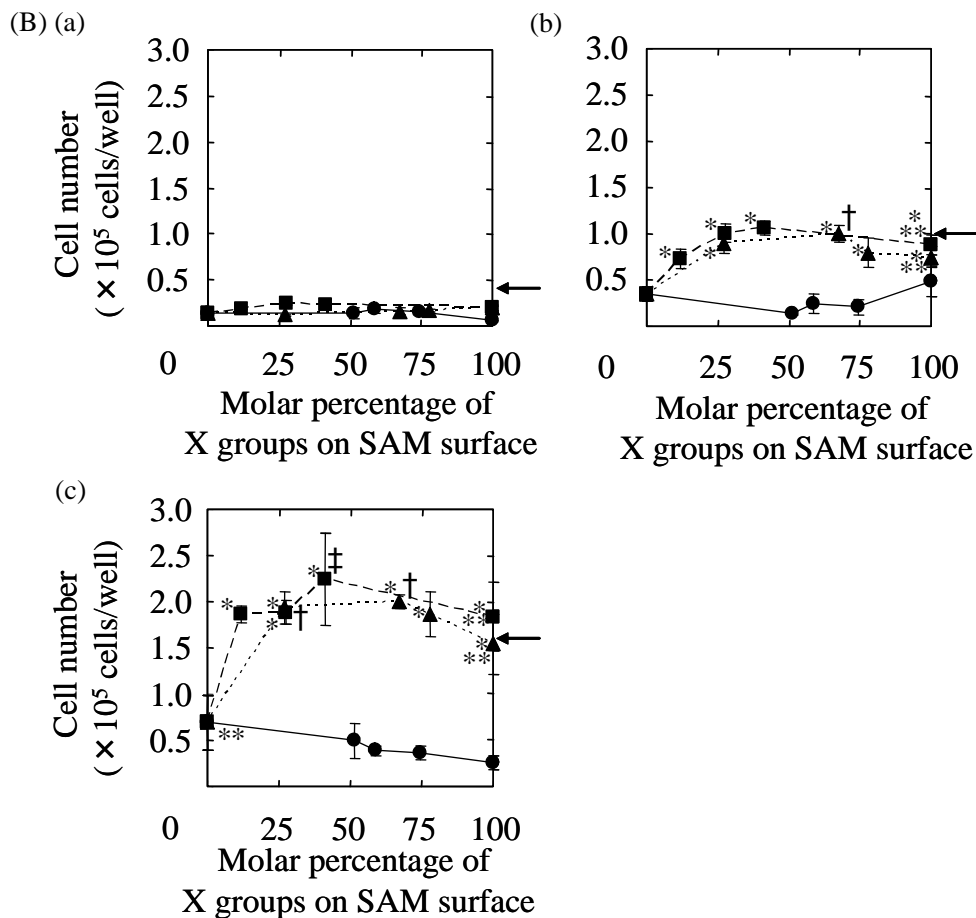
Figure 4. (Continued) (B) Number of fat-derived stem cells onto the SAM prepared at different OH;X mixing ratios : X=CH_3 (●), NH_2 (▲), and COOH groups (■) for 1 (a), 5 (b), and 9 days (c).
Molar percentage of X on the SAM surface was determined by XPS measurement. The arrow indicates the proliferation of fat-derived stem cells onto the cell culture dish.
*, $p<0.05$; significant against the proliferation of cells incubated onto the SAM of 100% OH group.
**, $p<0.05$; significant against the proliferation of cells incubated onto the SAM of 100% CH_3 group.
†, $p<0.05$; significant against the proliferation of cells incubated onto the SAM of 100% NH_2 group.
‡, $p<0.05$; significant against the proliferation of cells incubated onto the SAM of 100% COOH group.

Adipogenic differentiation of cells on SAM-modified substrates

Figure 5 (A) shows the phase-contrast micrographs of fat-derived stem cells cultured on different SAM or the cell culture dish in the ITT medium. When cultured in the ITT medium to differentiate into adipogenic cells, cells accumulated lipids inside the cells for every SAM sample. However, no lipid accumulation was observed in cell even after 30 days culture in the normal medium. Figure 5 (B) shows the GPDH activity of cells on different SAM 30 days after incubation in the ITT medium. The GPDH activity of cells cultured on the SAM of CH_3 and the mixed SAM of OH and COOH groups at the ratios of 88:12 and 73:27 was high compared with that of cells on SAM of OH group. The GPDH activity of cells on the cell culture dish was 3.0×10^7 IU/cell.
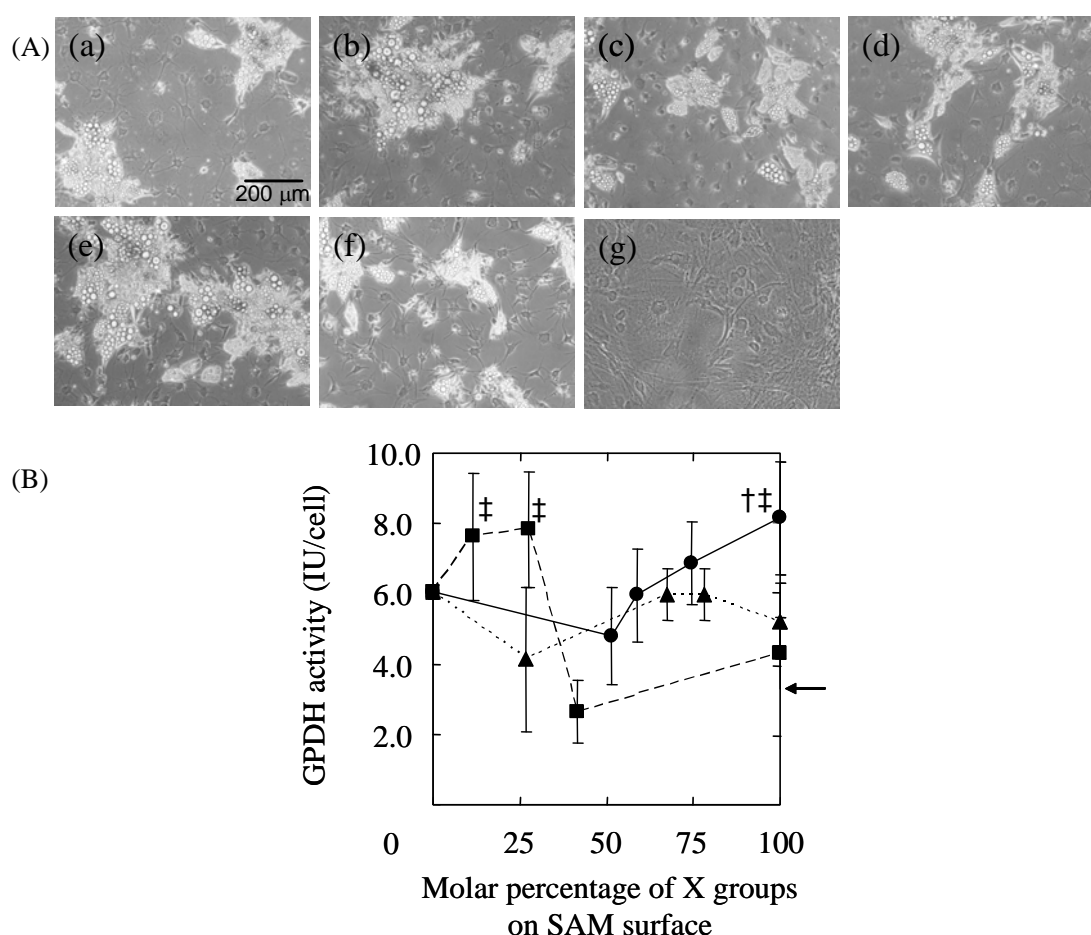
Figure 5. (A) Phase-contrast microscopic photographs of fat-derived stem cells incubated onto the SAM of OH (a), CH_3(b), NH_2 (c), COOH groups (d), and OH:COOH= 27:73 (e) or cell culture dish (f and g) for 1 day in the normal medium, followed by further for 30 days in the ITT medium (a-f) or normal medium (g). (B) GPDH activity of fat-derived stem cells incubated onto the SAM prepared at different OH;X mixing ratios : X=CH_3 (●), NH_2 (▲),and COOH groups (■)for 1 day in the normal medium, followed by further for 30 days in the ITT medium. The arrow indicates the GPDH activity of fat-derived stem cells onto the cell culture dish.
†, $p<0.05$; significant against the mRNA expression of cells incubated onto SAM of 100% NH_2 group.
‡, $p<0.05$; significant against the mRNA expression of cells incubated onto SAM of 100% COOH group.

Figure 5 (C) shows that the time course of PPARγ2 and aP2 mRNAs of cells cultured in the ITT medium. The level of PPARγ2 and aP2 mRNA increased 42-fold and 150-fold as high as that of cells cultured in the normal medium before induction of adipogenic differentiation and cells cultured on SAM of CH₃ group for 14 days. These levels were higher than those of cells cultured on the cell culture dish in the ITT medium. The PPARγ2 and aP2 mRNA levels did not change over the time range studied when cells were cultured in the normal medium.
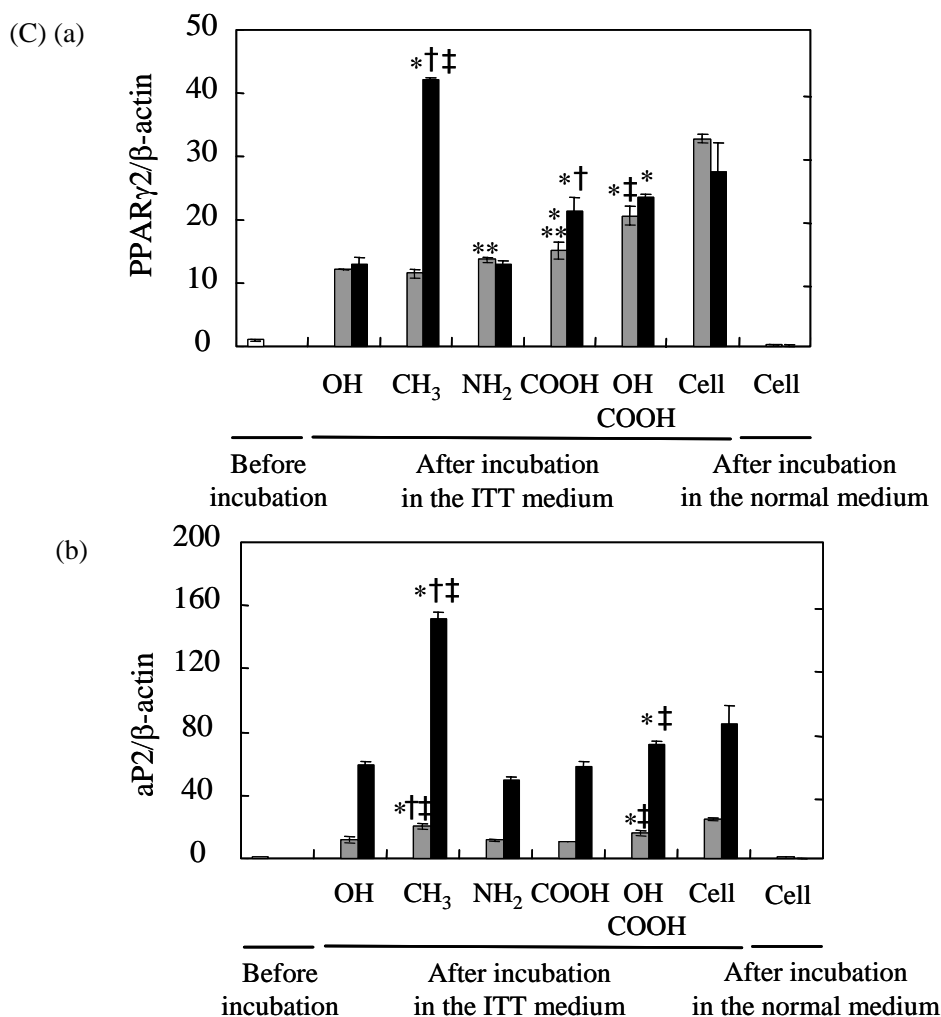
Figure 5. (Continued) (C) PPARγ2 (a) and aP2 expression (b) of fat-derived stem cells incubated onto SAM of OH, CH₃, NH₂, OH:COOH=73:27, and COOH groups or cell culture dish (Cell) for 1 day (□) in the normal medium, followed by further for 7 (▨) and 14 days (■)in the ITT medium and or normal medium.
Molar percentage of X on the SAM surface was determined by XPS measurement.
*, p<0.05; significant against the mRNA expression of cells incubated onto SAM of 100% OH group.
**, p<0.05; significant against the mRNA expression of cells incubated onto SAM of 100% CH₃ group.
†, p<0.05; significant against the mRNA expression of cells incubated onto SAM of 100% NH₂ group.
‡, p<0.05; significant against the mRNA expression of cells incubated onto SAM of 100% COOH group.

ERK phosphorylation of cells on SAM-modified substrates

Figure 6 shows phosphorylation of ERK1/2 in fat-derived stem cells. The incubation of fat-derived stem cells on the SAM of NH_2 and COOH groups for 8 hr increased the ERK1/2 phosphorylation of cells compared with the SAM of OH and CH_3 groups.

Figure 6. Phosphorylation of ERK1/2 in fat-derived stem cells incubated onto the SAM of OH, CH_3, NH_2, and COOH groups or the cell culture dish (Cell) : (A) immunoblotting patterns and (B) quantitative data.

Figure 7 (A) shows the suppression effect of ERK1/2 blockade with upstream kinase (mitogen-activated protein kinase (MAPK) kinase 1) inhibitor, PD98059 on the cell proliferation on the SAM of COOH groups and cell culture dish. PD98059 significantly inhibited the proliferation of cells. Figure 7 (B) shows the suppression effect of PD98059 on the expression level of PPARγ2 mRNA on COOH group. PD98059 induced the expression of PPARγ2 mRNA.
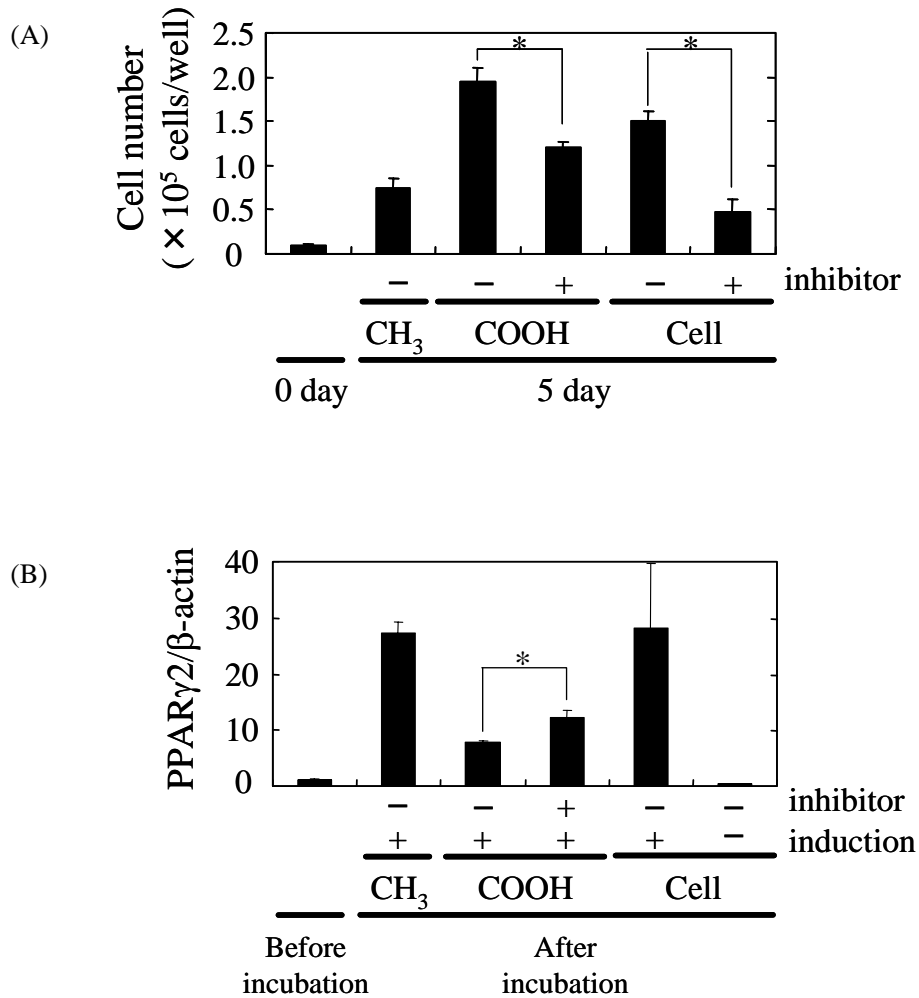
Figure 7. (A) Addition effect of ERK1/2 inhibitor on the proliferation of fat-derived stem cells. Fat-derived stem cells were incubated with or without 50 μM of PD98059 for 5 days onto the SAM surfaces.
*, p<0.05; significant against the proliferation of cells incubated with PD98059.
(B) Addition effect of ERK1/2 inhibitor on PPARγ2 expression of fat-derived stem cells. Fat-derived stem cells were incubated for 5 days onto the SAM surfaces in the normal medium (-) and ITT medium (+) with or without 50 μM of PD98059.
*, p<0.05; significant against the PPARγ2 expression of cells incubated with PD98059.

mRNA expression of cells on SAM-modified substrates

Figure 8 shows the level of CTGF and CYR61 mRNA 6 hr after incubation. The mRNA level of CTGF and CYR61 on the SAM of NH_2 and COOH groups was significantly higher than that on the SAM of CH_3 group.
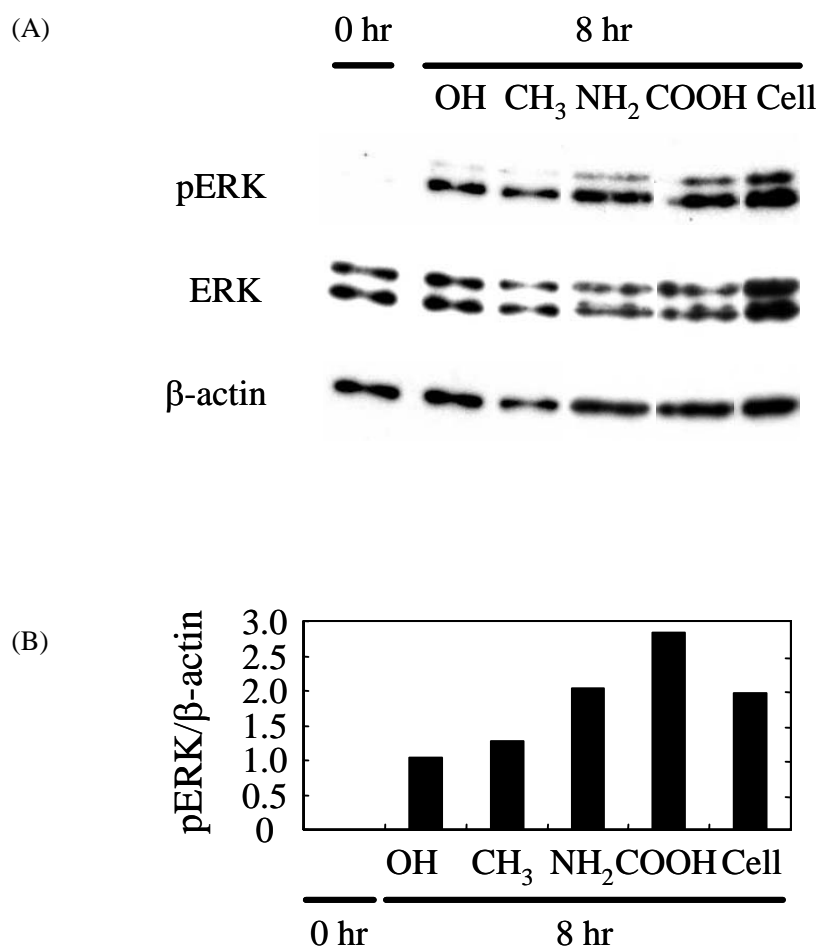
(A)

(B)
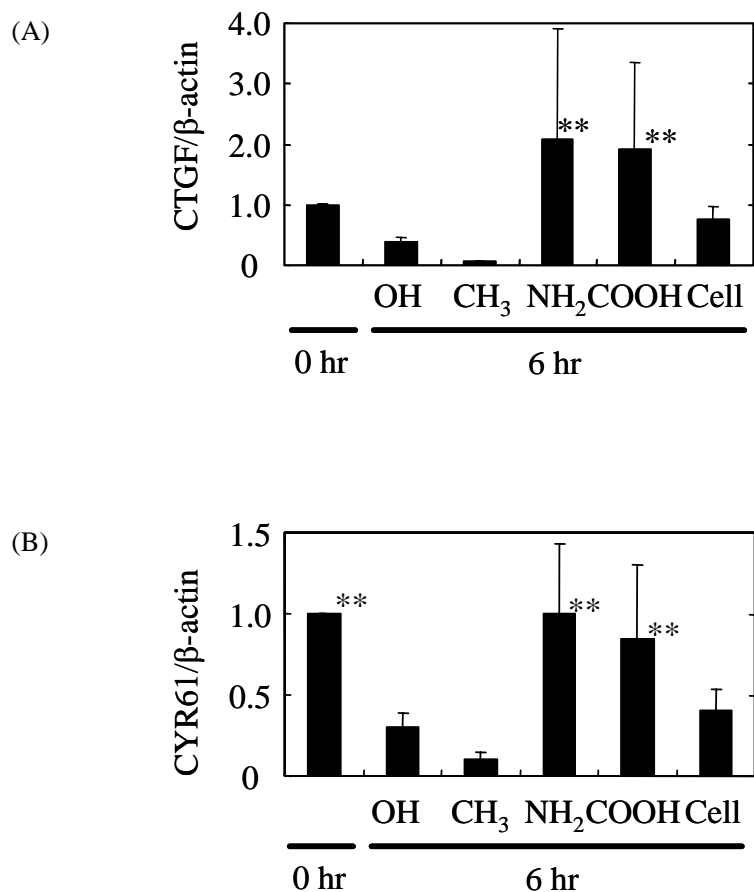
Figure 8 CTGF (A) and CRY61 (B) expression of fat-derived stem cells incubated onto the SAM of OH, CH_3, NH_2, and COOH groups or the cell culture dish (Cell).
**, $p<0.05$; significant against the mRNA expression of cells incubated onto SAM of 100% CH_3 group.

DISCUSSION

The technique of SAM has been extensively used to prepare cell culture substrates which have different chemical compositions of the surface [11-21]. The surface property of substrates affects protein adsorption onto the surface and the consequent cell attachment and proliferation. The present study demonstrate that the type of chemical groups to use the SAM formation greatly affected the attachment, proliferation, and adipogenic differentiation of fat-derived stem cells which were adult stem cells isolatable from patients. The cell behavior was evaluated from the viewpoint of the mRNA expression and an intercellular signaling activation.

In general, the SAM of alkanethiols are prepared on glass [12-15, 17, 19, 20] or silicon [18] after the surface coating of gold. In present experiment, a PET sheet was used as the substrate, because the PET sheet has been used as the cell culture substrate and the sheet is readily changed to fiber and mesh which may be interested as a 3-dimensional substrate. When compared between the SAM samples of glass and PET sheet, the contact angle was identical to each other for the surface of SAM which is prepared by the same alkanethiol (data not shown) [11, 14, 16, 18]. This indicates that PET surface also could be modified by the SAM technique and is usable for the purpose of present study. The SAM of alkanethiols have been widely used to evaluate effect of the chemically functional group on the behavior of cells [12-15, 17-20]. Unfortunately the SAM have often been prepared only by one type of functional group and such homogeneous SAM are limited from the viewpoint of research to investigate the interaction between cells and materials. Mixed SAM have been designed by using various types of alkanethiols, for example alkanethiols with different lengths of alkyl chain and functional groups [30, 31]. However, the mixed SAM have been hardly used to evaluate the surface influence on the cell behavior. In the present study, the SAM which have two functional groups on the surface were used for cell culture substrate. Generally, it is difficult to determine the chemical composition of SAM prepared in an ethanol solution containing two alkanethiols only by a simple equilibrium expression. Whitesides et al. showed that the composition of alkanethiols in ethanol solution was not always in agreement with the composition of alkanethiols on a SAM surface by analysis of XPS, although the molar percentage of an alkanethiol on the SAM surface increased with an increase of that in ethanol solution [30]. The composition of mixed SAM depended on the property of tail functional groups. In the present experiment, the composition of alkanethiols on the SAM surface was measured by XPS (Figure 1 (B)). On the mixed SAM of OH and NH_2 or OH and CH_3 groups, the molar percentage of NH_2 or CH_3 groups on the surface tended to be higher than that of the ethanol solution in preparation. On the other hand, the molar percentage of COOH group on the mixed SAM surface tended to be lower than that of the ethanol solution. The phenomenon can be explained in terms of the bulkiness

of COOH group. Since the COOH group is structurally bulky than the CH_3 and NH_2 groups, it is likely that the amount immobilized on the surface is lower than that fed initially due to the steric hindrance. The amount immobilized which was evaluated by the water contact angle and XPS measurements was different from that fed initially in ethanol solution (Figures 1 (A) and (B)). It is reported that the number of functional groups immobilized is not proportional to that of water contact angle measurement on the surface [32]. It is possible that the phenomenon happened in the SAM. Taken together, the molar percentage of X on the SAM surface was determined by the XPS measurement.

Adsorption of proteins onto a substrate is an important step for the subsequent cell adhesion to the substrate [33]. It is well recognized that several proteins present in FCS are adsorbed on the SAM surface, and the types and amount of proteins generally depend on the type of functional groups modifying the surface [11]. Cells generally are likely to adhere onto a substrate through the integrin receptors. The integrin receptor recognizes the RGDS sequence of cell adhesion moiety which is present in vitronectin and fibronectin molecules [34]. Faucheux et al. showed that adsorption of vitronectin in FCS on the NH_2 surface was higher than that on CH_3 and COOH surfaces when the adsorption was measured by the western blotting method [11]. Keselowsky et al. showed that fibronectin was adsorbed onto the CH_3, COOH, and NH_2 surfaces to a higher extent than the OH surface using radiolabeled fibronectin [14]. The orientation of fibronectin adsorbed on the SAM surfaces is examined by use of the monoclonal antibody, which can recognize the cell-binding domain of fibroncetin [14]. Binding affinity of the antibody to fibronectin adsorbed on the SAM surface was significantly different (NH_2=CH_3=COOH>OH). In the present experiment, although vitronectin and fibronectin in FCS adsorbed on SAM surfaces were measured by the modified ELISA method. The result of vitronectin adsorption was in good agreement with that reported previously, while the adsorption profile of fibronectin was different from that of previous study (Figures 2 (A) and (B)). The difference in the protein adsorption might due to the condition of adsorption experiments. The fibronectin adsorption from the solution was examined in the previous study, but in the present study done from FCS. After all, the adsorptions of vitronectin and fibronectin did not affect the morphology of cells attached. Keselowsky et al. investigate the effect of fibronectin density on the amount of integrin receptor [14]. The amount of integrin did not increase with an increase in the fibronectin density. This indicates that the cell adhesion is affected not only by the amount of protein adsorbed on the surface of SAM, but also by other factors, such as the orientation of proteins on the surface. It is possible that the spreading of fat-derived stem cells on the SAM is affected by the molecular orientation of vitronectin and fibronectin adsorbed. When modified by the SAM method, the surface had a island structure of alkanethiol with a nano-order size [35]. Thus, the mixed SAM technology sometimes results in the

segregation of micro/nano domains, which could affect the protein adsorption and the subsequent cellular responses. This structural feature should be considered to use the SAM technology for surface modification.

SAM have been applied as the culture substrate for various types of cells [11-21]. The spreading and proliferation of fat-derived stem cells on the SAM of NH_2 and COOH groups were different from those of CH_3 SAM, which is in good accordance with results reported for other cells [11, 16]. The NH_2 and COOH substrates have a positive or negative charge which affects the amount and orientation of protein adsorbed on the surface [14]. The feature causes the high level of cell adhesion. The spreading and proliferation of fat-derived stem cells were high for the mixed SAM of OH and NH_2 or OH and COOH groups. Although the surface charge of mixed SAM was smaller than that of respective SAM of NH_2 or COOH groups, the charge would be large enough for fat-derived stem cells to recognize the substrates [14]. Cell spreading is one of the events induced by the biological interaction between the integrin receptor and a substrate [34]. Cells contact the surface of substrates while the substrate-integrin interaction occurs. In this case, the cells begin to flatten and their plasma membrane spread, leading to the actin organization and the occurrence of focal adhesion. This is confirmed by Figures 3 (A) and (B). Following the focal adhesion, it is reported that a lot of intercellular signaling events take place, including the activation of focal adhesion kinase, phosphatidylinositol 3-kinase, and MAPK. The signaling regulates the consequent the cell migration, survival, and proliferation [27, 34, 36]. ERK1/2 is a MAPK which has a key influence on cell proliferation [25]. As shown in Figure 6, the level of ERK1/2 activation was different among the SAM. SAM which induced the activation of ERK1/2 were similar to those of enhanced proliferation of cells (Figure 4 (B)). The cell proliferation was changed by treatment with ERK1/2 inhibitor (Figure 7 (A)). Taken together, it is possible that fat-derived stem cells attached on the SAM surface recognizing the difference in the surface properties. As the result, it is possible that the actin organization and the level of ERK1/2 activation were changed, while the change affected the pattern of cell proliferation.

Adipogenic differentiation of fat-derived stem cells was different among the SAM. The adipogenic differentiation of fat-derived stem cells was high on the SAM of CH_3 group which inhibited cell spreading (Figure 3 (B)) and proliferation (Figure 4 (B)). A micropattern technique can investigate the relation between cell spreading and cell differentiation. Adipogenic differentiation of MSC was observed upon incubating in a mixed adipogenic and osteogenic medium on a small island surface which inhibited the cell spreading [37]. Generally, the less cell proliferation is, the higher cell differentiation is [38]. It is possible that suppressed spreading and proliferation of cells resulted in their higher differentiation. A recent research demonstrates that MAPK regulates adipogenic gene expression [39]. The SAM of COOH group activated ERK1/2

and inhibited the adipogenic differentiation compared with those of CH_3 (Figures 5 (B) and 6). For the SAM of COOH group, the adipogenic differentiation of cells was enhanced when cells was treated with the ERK1/2 inhibitor (Figure 7 (B)). It is conceivable that diminished signaling via ERK1/2 on the SAM of CH_3 group is the reason why adipogenic differentiation of fat-derived stem cells was enhanced on the SAM of CH_3 group. Fat-derived stem cells on the mixed SAM of OH and COOH groups showed oil lipids accumulation in the cells and a high GPDH activity compared with those on the SAM of COOH group (Figures 5 (A) and (B)). The adipogenic differentiation depends on the expression of PPARγ2. The level of mRNA expression on the mixed SAM of OH and COOH groups was high (Figure 5 (C)). For the mixed SAM of OH and COOH groups, the chemical composition dependence of GPDH activity is similar to that of fibronectin adsorption (Figure 2 (B)). The mixed SAM surface of OH and COOH groups gives cells a biological stimulus by way of the integrin receptor. It is possible that the integrin signal consequently increases the level of mRNA expression, resulting in induction of adipogenic differentiation. Although the adipogenic differentiation was enhanced on the SAM of CH_3 group, it was low on the SAM of OH and NH_2 groups. It is reported that human MSC on the SAM of NH_2 group expressed mRNA which is related to osteogenic differentiation [21]. Fat-derived stem cells also have multi differentiation potentials similarly to the MSC [1-3]. The SAM of OH and NH_2 groups might be suitable for differentiation of fat-derived stem cells other than adipogenesis.

CTFG and CYR61 are the members of CCN (CYR61, CTGF, and Nov) family which play an important role in the cell proliferation [23]. The mRNA expression of CTGF and CYR61 was high for the SAM of NH_2 and COOH groups (Figure 8). The surface composition of substrate controlled mRNA expression of cells on the surface. The SAM of NH_2 and COOH groups induced the proliferation of fat-derived stem cells (Figure 4 (B)). It is possible that the higher level of CCN family expressed results in the higher proliferation of fat-derived stem cells.

REFERENCES

1. P. A. Zuk, M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz and M. H. Hedrick *Tissue Eng* **7**, 211 (2001)

2. P. A. Zuk, M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim and M. H. Hedrick *Mol Biol Cell* **13**, 4279 (2002)

3. K. M. Safford, K. C. Hicok, S. D. Safford, Y. D. Halvorsen, W. O. Wilkison, J. M. Gimble and H. E. Rice *Biochem Biophys Res Commun* **294**, 371 (2002)

4. T. Yoshikawa, H. Ohgushi and S. Tamai *J Biomed Mater Res* **32**, 481 (1996)

5. A. S. Goldstein, G. Zhu, G. E. Morris, R. K. Meszlenyi and A. G. Mikos *Tissue Eng* **5**, 421 (1999)

6. K. Yamanouchi, K. Satomura, Y. Gotoh, E. Kitaoka, S. Tobiume, K. Kume and M. Nagayama *J Bone Miner Res* **16**, 857 (2001)

7. H. A. Awad, M. Q. Wickham, H. A. Leddy, J. M. Gimble and F. Guilak *Biomaterials* **25**, 3211 (2004)

8. Y. Hori, S. Inoue, Y. Hirano and Y. Tabata *Tissue Eng* **10**, 995 (2004)

9. S. Inoue, Y. Hori, Y. Hirano, T. Inamoto and Y. Tabata *J Biomater Sci Polym Ed* **16**, 57 (2005)

10. A. Ulman *Chem Rev* **96**, 1533 (1996)

11. N. Faucheux, R. Schweiss, K. Lutzow, C. Werner and T. Groth *Biomaterials* **25**, 2721 (2004)

12. S. Zhang, L. Yan, M. Altman, M. Lassle, H. Nugent, F. Frankel, D. A. Lauffenburger, G. M. Whitesides and A. Rich *Biomaterials* **20**, 1213 (1999)

13. R. Daw, I. M. Brook, A. J. Devlin, R. D. Short, E. Copper and G. J. Leggett *J Mater Chem* **8**, 2583 (1998)

14. B. G. Keselowsky, D. M. Collard and A. J. Garcia *J Biomed Mater Res A* **66**, 247 (2003)

15. B. G. Keselowsky, D. M. Collard and A. J. Garcia *Biomaterials* **25**, 5947 (2004)

16. P. Filippini, G. Rainaldi, A. Ferrante, B. Mecheri, G. Gabrielli, M. Bombace, P. L. Indovina and M. T. Santini *J Biomed Mater Res* **55**, 338 (2001)

17. C. A. Scotchford, C. P. Gilmore, E. Cooper, G. J. Leggett and S. Downes *J Biomed Mater Res* **59**, 84 (2002)

18. J. N. Barbosa, M. A. Barbosa and A. P. Aguas *J Biomed Mater Res A* **65**, 429 (2003)

19. M. Franco, P. F. Nealey, S. Campbell, A. I. Teixeira and C. J. Murphy *J Biomed Mater Res* **52**, 261 (2000)

20. Y. Naka, A. Eda, H. Takei and N. Shimizu *J Biosci Bioeng* **94**, 434 (2002)

21. J. M. Curran, R. Chen and J. A. Hunt *Biomaterials* **26**, 7057 (2005)

22. Y. Tamada and Y. Ikada *in: Polymer in Medicine II* p. 101. (1986)

23. B. Perbal *Mol Pathol* **54**, 57 (2001)

24. A. J. Garcia, M. D. Vega and D. Boettiger *Mol Biol Cell* **10**, 785 (1999)

25. Q. L. Cui and G. Almazan *J Neurochem* **100**, 1480 (2007)

26. H. Hauner, G. Entenmann, M. Wabitsch, D. Gaillard, G. Ailhaud, R. Negrel and E. F. Pfeiffer *J Clin Invest* **84**, 1663 (1989)

27. Y. T. Lin, C. H. Tang, W. J. Chuang, S. M. Wang, T. F. Huang and W. M. Fu *Biochem Pharmacol* **70**, 1469 (2005)

28. J. Rao and W. R. Otto *Anal Biochem* **207**, 186 (1992)

29. M. F. Simon, D. Daviaud, J. P. Pradere, S. Gres, C. Guigne, M. Wabitsch, J. Chun, P. Valet and J. S. Saulnier-Blache *J Biol Chem* **280**, 14656 (2005)

30. C. D. Bain, J. Evall and G. M. Whitesides *J Am Chem Soc* **111**, 7155 (1989)

31. C. D. Bain and G. M. Whitesides *J Am Chem Soc* **111**, 7164 (1989)

32. Y. Ikada *Biomaterials* **15**, 725 (1994)

33. M. Balcells and E. R. Edelman *J Cell Physiol* **191**, 155 (2002)

34. U. Hersel, C. Dahmen and H. Kessler *Biomaterials* **24**, 4385 (2003)

35. G. E. Poirier *Chem Rev* **97**, 1117 (1997)

36. W. Guo and F. G. Giancotti *Nat Rev Mol Cell Biol* **5**, 816 (2004)

37. C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides and D. E. Ingber *Science* **276**, 1425 (1997)

38. R. McBeath, D. M. Pirone, C. M. Nelson, K. Bhadriraju and C. S. Chen *Dev Cell* **6**, 483 (2004)

39. F. Bost, M. Aouadi, L. Caron and B. Binetruy *Biochimie* **87**, 51 (2005)

Chapter 3

Adipogenic differentiation of human fat-derived stem cells cultured with bFGF in the solution and coated form

INTRODUCTION

It has been reported that the biological action of growth factors and cytokines on cells depends upon the existence mode in the cell culture. Various types of growth factors and cytokines, such as epidermal growth factor (EGF) [1-3], brain-derived neurotrophic factor (BDNF) [4], nerve growth factor (NGF) [4], bFGF [5], and insulin [6-8], were immobilized onto the culture substrate of cells through the physical adsorption [3, 5] and chemical coupling methods by photo-irradiation [4, 7] and chemical crosslinking [1, 2, 6, 8]. When evaluated in terms of the signaling pathway, the activity of growth factors and cytokines in the immobilized form was found to be different from that present in the culture medium [2, 3, 7]. The consequent spreading [3], adhesion [3], proliferation [1, 2, 6-8] or osteogenic differentiation of cells [5] and neuron outgrowth [4] are greatly influenced by the existence mode of growth factors and cytokines in the cell culture.

Fat-derived stem cells which can be isolated from the adipose tissue are capable of differentiating into adpogenic, osteogenic, chondrogenic, neurogenic, and myogenic cells [9-12]. When added into an adipogenic differentiation medium for cell culture, bFGF inhibited the adipogenic differentiation of fat-derived stem cells [13]. On the other hand, when the cells were evaluated on the culture dish coated with bFGF, the adipogenic differentiation was inhibited [14]. However, there is no research to directly compare the bFGF action on the cell adipogenic differentiation between the existance modes of solution and immobilization. bFGF binds the receptors on the cell membrane, which activated the subsequent signaling pathway inside cells, such as ERK1/2 [15]. In addition, it is demonstrated that the ERK1/2 activation affects the gene expression which is related to the adipogenic differentiation of cells [16].

This study was undertaken to investigate the effect of bFGF in the solution and coated forms on the adhesion and adipogenic differentiation of fat-derived stem cells. We also examine the profile of ERK1/2 activation to evaluate the dependence on the behavior of cells cultured.

EXPERIMENTAL

Isolation and culture of human fat-derived stem cells

Fat-derived stem cells were primarily isolated from the human fat tissue which had been collected in the reduction mammaplastic surgery of breast cancer patients after obtaining the informed consent at Kyoto University Hospital. The fat tissue (5 ml) was washed with 100 mM of PBS, pH7.4 to carefully remove blood cells, then minced, and digested by 520 U/ml collagenase (Nitta Gelatin Inc., Osaka, Japan) at 37 ^{o}C for 15 min under shaking [14]. The digested was suspended in Medium 199 containing 10 vol% fetal calf serum (FCS, #AJL12371, Hyclone Laboratories Inc., UT) and 1 vol% penicillin-streptomycin (normal medium), followed by the filtration through a 200-μm nylon mesh and centrifugation at 1,000 rpm for 5 min at 4 ^{o}C. After washing twice with the normal medium, the cells obtained were again suspended in the normal medium and cultured at 37 ^{o}C in a 75-cm^2 culture flask (#430720, Corning Inc., NY). Non-adherent cells were removed 1 day later and adherent cells were proliferated in the control medium to use the following experiments.

To exclude the FCS influence on cells, the fat-derived stem cells cultured were washed with PBS at a middle confluent state and incubated in Medium 199 containing 0.2 wt% bovine serum albumin (FCS-free medium) for 3 days [7, 15]. The cells were detached with 0.05 wt% trypsin solution-containing 0.2 wt% ethylenediamineteraacetic acid in PBS. Trypsin inhibitor (#030K70191, type II-S soybeans, Sigma Aldrich Co., MO., 1 mg/ml) in the FCS-free medium was added to inhibit trypsin activity. After centrifugation at 1,000 rpm for 5 min at 4 ^{o}C, cells were suspended into the FCS-free medium. To prepare the culture dish coated with bFGF, human recombinant bFGF, kindly supplied by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan in PBS (2.5 ng/ml) was added into a cell culture dish (0.25 ng/cm^2) at a volume of 100 μl per cm^2 dish, left at 37 ^{o}C for 1 hr, and washed with PBS to exclude bFGF un-adsorbed. The amount of bFGF adsorbed (coated) on the cell culture dish was 0.49 ng per dish (2 cm^2, 24-well multiwell dish, #08607024, Corning Inc.) when calculated from the calibration curve between the amounts of bFGF adsorbed and added for dish coating [5]. Then, the cells were plated onto the dish coated with bFGF at a density of 1×10^4 cells/cm^2 dish. On the other hand, 500 μl of cell suspension containing 1 ng/ml bFGF was plated on a cell culture dish. The total amount of bFGF (0.5 ng/dish) was similar in both the coated and medium solution forms.

For adipogenic differentiation, the fat-derived stem cells were cultured in DME/Ham's F12 medium (250 μl/cm^2) containing 0.05 μM insulin, 0.2 nM 3,5,3'-triiodothyronine, 100 nM transferrin, 17 μM calcium pantotenate, 33 μM biotin, and 100 nM dexamethasone (ITT medium) [13]. The cells were initially plated at a density of 5×10^4 cells/cm^2 and cultured on the culture dish

in the ITT medium containing bFGF or on the culture dish coated with bFGF in the ITT medium. The corresponding fresh ITT medium containing bFGF was exchanged every 3 days for cell culture with bFGF in the coated and solution medium forms, respectively.

Evaluation of cell morphology and number

The morphology of cells 3 hr after plating was observed by a phase-contrast microscopy (IX70, Olympus Optical Co., Ltd., Tokyo, Japan). The number of cells was determined by a DNA assay method [17]. Briefly, the cells were incubated in 1 ml of aqueous solution containing 0.2 mg/ml sodium dodecyl sulfate, 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate for 1 hr for cell lysis. The cell lysate (100 μl) was mixed 100 μl solution of 1 μl/ml Hoechst 33258 dye solution (bisbenzimide H33258 fluorochrome trihydrochloride DMSO solution, #L2E4809, Nacalai Tesque, Inc., Kyoto, Japan), 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate, and then the fluorescence intensity of mixed solution was measured on a fluorescence spectrophotometer (Spectra Max Gemini Em, Molecular Device Japan Co., Osaka, Japan) at exciting and emission wavelengths of 355 and 460 nm. The cell number was determined by use of the calibration curve prepared by the cells of known number. The experiment was done two times independently, otherwise mentioned.

Immunobloting of ERK phosphorylation

After 4 and 8 hr culturing in the FCS-free medium containing bFGF or the cell culture dish coated with bFGF, fat-derived stem cells were lysed with a Radio-Immunoprecipitation Assay buffer containing protease inhibitors (#065K4070, Sigma Aldrich Co.). The protein content of cell lysates was measured using the BioRad Protein Assay Kit (BioRad Laboratories Inc., CA). The cell lysates underwent the sodium dodecylsulfate polyacrylamide electrophoresis on a 12 vol% gel, followed by the transferring to an Immobilon-P membrane (#K6PN7152C, Millipore Co., MA) [18]. The membrane was immunoblotted with primary antibodies against phospho-ERK1/2 (1:1000) (#9101S, Cell Signaling Technology Inc., MA) and β-actin (1:10000) (#813K4879, Sigma Aldrich Co.) of an internal control. After treatment with peroxidase-conjugated secondary antibodies (anti-mouse; #HA9825214, and anti-rabbit; #FK928983, Thermo Fisher Scientific Inc., IL) the immunoreactive proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (#H5106367, Thermo Fisher Scientific Inc.).

Real time RT-PCR

RNA was isolated from cells using the RNeasy Plus Mini Kit (#7272462, Qiagen Co., CA) according to the manufacture's instructions [18]. Reverse transcription reaction was performed with the SuperScript Frist-Stand Synthesis System (Invitrogen Co., CA). Real time PCR was

performed on the 7500 real time PCR system (Applied Biosystem Japan Ltd., Tokyo, Japan) for 10 ng of cDNA in a total volume of 25 μl containing SYBR Green PCR Master Mix (Applied Biosystem Japan Ltd.) and 10 μM of each primer (Table 1). The reaction mixture was incubated for the initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles.　Each cycle consisted of the following three steps; 94 °C for 15 sec, 57 °C for 15 sec, and 72 °C for 1 min. Each mRNA level was normalized by an internal β-actin control.

Table 1. Primers used for real time RT-PCR assays

Name	Primer	
PPARγ2	sense	GAAAGCGATTCCTTCACTGATACA
	antisense	AATAAGGTGGAGATGCAGGCTC
aP2	sense	TACTGGGCCAGGAATTTGAC
	antisense	TGGTTGATTTTCCATCCCAT
β-actin	sense	ATGGTGGGCATGGGTCAGAAGG
	antisense	ACGCACGATTTCCCGCTCGGCC

Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity

The GPDH activity was assayed by a commercial kit (GPDH activity measurement kit, #JFL003, Sangi Co., Ltd., Tokyo, Japan) [13]. Cells were rinsed twice with PBS and homogenized in a buffer solution of kit using a handy sonic (UR-20, Tomy Seiko Co., Ltd., Tokyo, Japan) on ice. After mixing a reaction solution of kit, the absorbance of solution mixture was measured at 340 nm by the spectorophotometer (Versa max, Molecular Device Japan Co.) to assess the GPDH activity. The cell number was measured by a DNA assay method to normalize the GPDH activity.

Cell staining

The cells incubated in the ITT medium for 14 days were rinsed twice with PBS and fixed with 10 vol% formalin solution in PBS for 1 hr at 4 °C. Following rinsed twice with PBS and 60 vol% isopropanol in DDW, they were stained with an oil red O solution for 30 min at room temperature. The oil red O solution was prepared by mixing an oil red O (0.3 wt%) stock solution in isopropanol (6 ml) with 4 ml of DDW for 10 min at room temperature, followed by the filtration through a 0.22-μm filter (Millipore Co.) [14]. The cells were rinsed with the isopropanol aqueous solution to view by a phase-contrast microscopy (IX70, Olympus Optical Co., Ltd).

Data analysis

All the data were statistically analyzed by the post-hoc Fisher's PLSD test and expressed as the mean ± the standard deviation of the mean. Statistical significance was accepted at $p < 0.05$.

RESULTS

Adhesion of cells cultured with bFGF in the solution and coated forms

Figures 1 (A) and (B) show the morphology and number of fat-derived stem cells after 3 hr incubation in FCS-free medium with or without bFGF. For both the cultures in the FCS-free medium containing bFGF and on the dish coated with bFGF, cells adhered onto the culture dish. The morphology of cells was similar to that of the cell culture dish without bFGF. No difference in the number of cells adhered was observed, irrespective of the bFGF presence and the existence mode. The bFGF coating on the cell culture dish had no influence on the cell adhesion in terms of cell morphology and number.
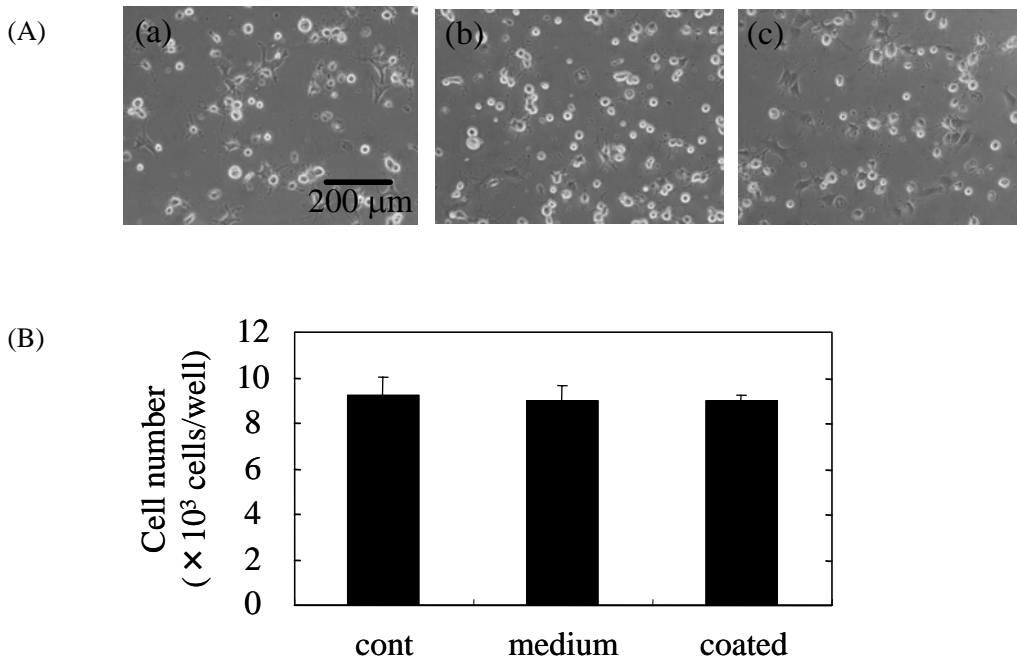

Figure 1. (A) Phase-contrast microscopic photographs of fat-derived stem cells incubated for 3 hr on the culture dish in the FCS-free medium (cont) (a) and that containing bFGF (medium) (b) or on the cell culture dish coated with bFGF in the FCS-free medium (coated) (c). (B) Number of adipo-stromal cells adhered after 3 hr incubation for the corresponding group shown in (A).

ERK phosphorylation of cells cultured with bFGF in the solution and coated forms

.   Figure 2 shows the ERK1/2 phosphorylation of fat-derived stem cells cultured in the FCS-free medium with or without bFGF and on the culture dish. The bFGF presence enhanced the phosphorylation of ERK1/2. The enhanced level was high in the FCS-free medium containing bFGF compared with that of bFGF in the coated form, irrespective of the culturing time. On the contrary, in the absence of bFGF, the ERK1/2 phosphorylation was not observed.
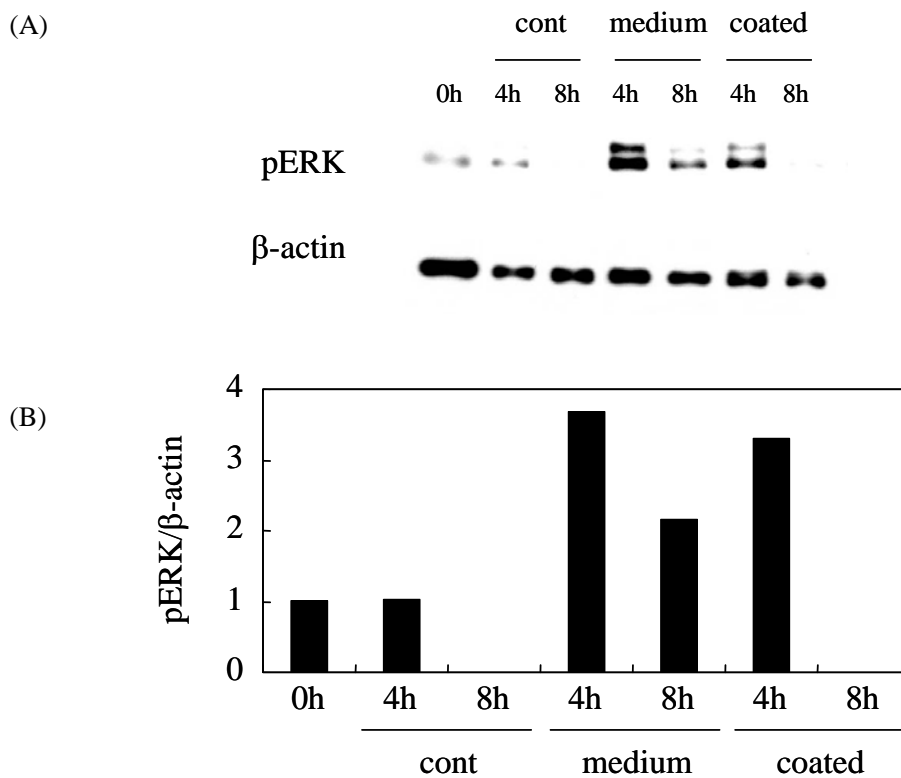

Figure 2. Phosphorylation of ERK1/2 in fat-derived stem cells incubated on the culture dish for 0, 4, and 8 hr in the FCS-free medium (cont) and that containing bFGF (medium) or on the cell culture dish coated with bFGF in the FCS-free medium (coated): (A) immunoblotting and (B) the quantitative results.

Adipogenic differentiation of cells cultured with bFGF in the solution and coated forms

        Figure 3 shows the mRNA expression of PPARγ2 and aP2 for cells cultured in the ITT medium. Incubation with the ITT medium increased the expression of PPARγ2 and aP2 mRNA to a significantly higher extent than that of the FCS-free medium. The mRNA expression was suppressed by bFGF in the solution form, contrast with that in the coated form. Figure 4 shows the GPDH activity of cells after incubation for 14 days in the FCS-free medium and ITT medium. The

GPDH activity of cells incubated in the ITT medium was significantly higher than that in the FCS-free medium. The bFGF presence in the solution or coated form decreased the GPDH activity at a similar level. Figure 5 shows the phase-contrast microscopic photographs of cells oil red O-stained. For culturing in the FCS-free medium, no cells accumulated oil lipids. However, the cell culture in the ITT medium increased the rate of cells stained. Irrespective of the existence mode of bFGF, the number of oil lipids seemed to decrease by bFGF addition.
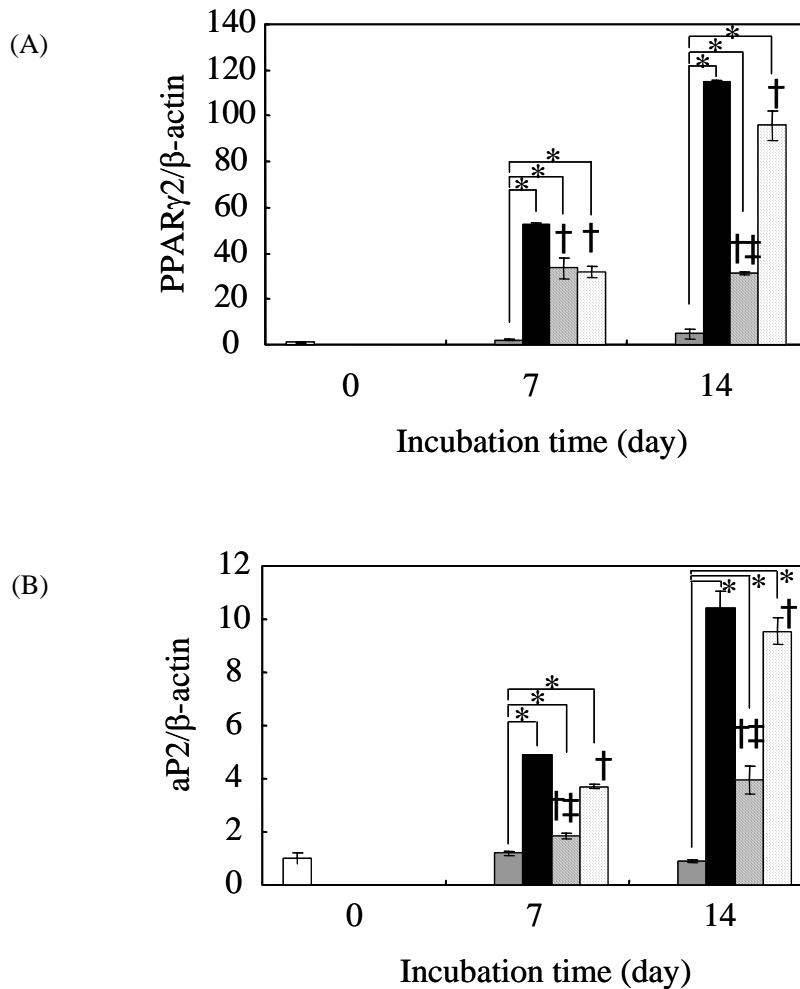
Figure 3. PPARγ2 (A) and aP2 (B) expression of fat-derived stem cells before (□), and after incubation for 7 and 14 days on cell culture dish in the FCS-free medium (▦), in the ITT medium (■), and that containing bFGF (▨)or on the cell culture dish coated with bFGF in the ITT medium (▱).
*, $p < 0.05$: significant against the mRNA expression of cells incubated in the FCS-free medium.
†, $p < 0.05$: significant against the mRNA expression of cells incubated in the ITT medium.
‡, $p < 0.05$: significant against the mRNA expression of cells incubated on the cell culture dish coated with bFGF in the ITT medium.
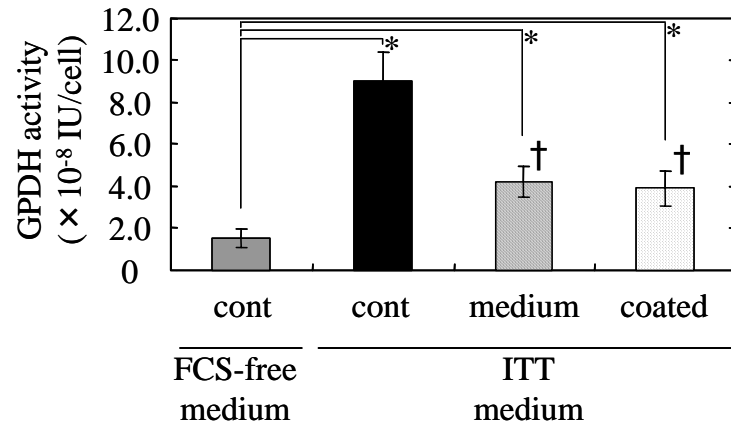
Figure 4. GPDH activity of fat-derived stem cells incubated for 14 days on the culture dish in the FCS-free medium (cont), in the ITT medium (cont), and that containing bFGF (medium) or on the cell culture dish coated with bFGF in the ITT medium (coated).

*, $p<0.05$: significant against the mRNA expression of cells incubated in the FCS-free medium.

†, $p<0.05$: significant against the mRNA expression of cells incubated in the ITT medium.

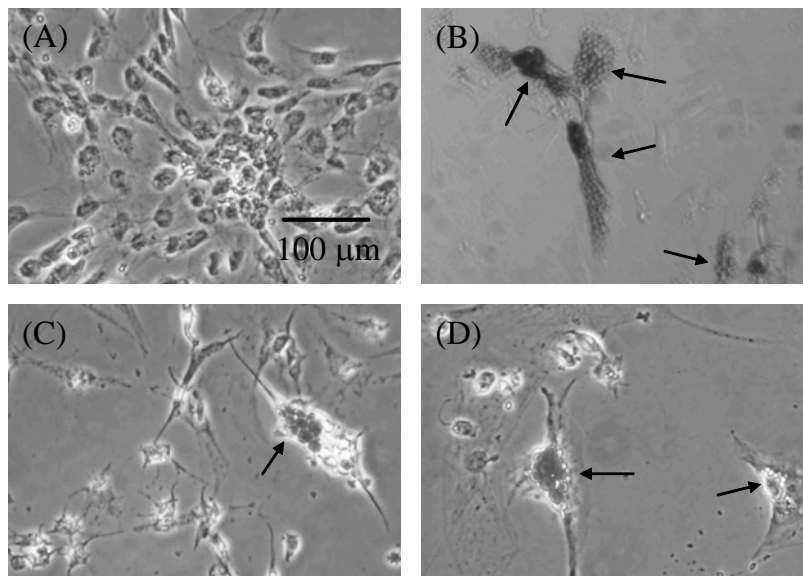Figure 5. Phase-contrast microscopic photographs of fat-derived stem cells stained by oil red O. The cells were incubated for 14 days on the cell culture dish in the FCS-free medium (A), in the ITT medium (B), and that containing bFGF (C) or on the cell culture dish coated with bFGF in the ITT medium (D). The arrow indicates oil lipids accumulated in the cell.

DISCUSSION

Growth factors induce the activation of cell signaling pathway after their binding the receptors. There are four action modes of growth factors to bind the receptor, such as juxtacrine, autocrine, paracrine, and endocrine fashions [19]. The juxtacrine fashion is that a growth factor anchored on the plasma membrane of cells or extracellular matrix acts on the cell receptor. The autocrine fashion is that cells produce a growth factor to act on the receptor for themselves. The paracrine or endocrine fashion is that a growth factor acts on the receptor of cells near or far through the diffusion in the tissue or blood circulation. Immobilization of growth factors is a mode of juxtacrine fashions and addition of the growth factors in the medium is a mode of paracrine or endocrine fashions. It is demonstrated that the action mode of growth factors have an influence on their biological functions [19]. Growth factors, EGF [1-3], BDNF [4], NGF [4], bFGF [5], and insulin [6-8], showed different actions of the spreading [3], adhesion [3], proliferation [1, 2, 6-8] or osteogenic differentiation of cells [5] and neuron outgrowth [4] by the existence mode, such as the addition into the culture medium and on the cell culture substrate. However, no effect of bFGF on the fat-derived stem cells has been studied.

When cells contact a substrate surface, they begin to flatten and the plasma membrane spreads [20], leading to the actin organization. It was found that the action mode of EGF affected the manner of actin organization. The filopodia were formed on the EGF-immobilized dish, while the presence of EGF in the culture medium induced the formation of lammellipodia [3]. bFGF in the solution or coated form did not affect the morphology of cells spread. It is known that the actin organization is influenced by cell signaling pathway [21]. Further investigation will be needed to discuss the bFGF effect on the cell morphology in terms of cell signaling pathway.

Culture of PC12 cells on the EGF-immobilized culture substrate activated the ERK and p38 for longer time periods than that of EGF added in the culture medium [2]. The prolonged ERK and p38 activation by the immobilized EGF was also observed for A431 cells [3]. When CHO-T cells were incubated with insulin in the immobilized or medium solution form, the phosphorylation of insulin receptor and the phosphatidylinositol-3-kinase activity continued to increase over 12 hr for the insulin immobilized, in contrast to the solution insulin [7]. As apparent from Figure 2, bFGF in the solution form activated ERK at a higher level and for a longer time period than that in the coated form. The difference in the ERK activation of fat-derived stem cells by bFGF can be explained in terms of the nature of bFGF and cells. The first reason is the difference in the density of coated. For bFGF, the receptor dimerization is required for the signal transduction [22]. If the density of bFGF coated is too low to induce the receptor dimerization, the activation of cell signaling pathway will be decreased. For example, baby hamster kidney (BHK) 21 cells have

60,000 bFGF receptors per cell to bind bFGF [23]. On the assumption that the area of one cell spread is 600-1000 μm^2 which is calculated by a computer analysis, the density of bFGF receptor is 6×10^9-1×10^{10} receptor/cm^2. In this study, since the coating density of bFGF was 0.25 ng/cm^2, the molecular density is 8×10^{10} ligand/cm^2 base on the molecular weight of bFGF [24]. Therefore, it is possible that the number of bFGF adsorbed was enough to bind the receptors. The second reason is the denaturation of bFGF coated. Generally, it is recognized that protein is denatured when directly coated onto the surface of solids [25]. To prevent the denaturation of bFGF, heparin and fibrin are used for immobilization of bFGF [26, 27]. In this study, heparin was not used because it often functions as an inhibitor of bFGF action, although effectively suppresses the denaturation [28]. When L929, BKH 21, and fat-derived stem cells were cultured with various concentrations of bFGF in the solution or coated form, the proliferation rate of cells cultured with bFGF in the coated form was as same as or lower than that in the solution form. In our previous study, it is demonstrated that the concentration of bFGF in the coated form needed to grow was 1,000 times higher than that of bFGF in the solution form. It is highly conceivable that bFGF molecules directry coated are denatured in a certain extent. This denaturation would result in suppressed biological activity of bFGF. The third reason is the nature of fat-derived stem cells. The immobilized EGF enhanced the proliferation of CHO-K1 cells and STO cells to a great extent compared with EGF added into the medium. On the other hand, EGF in the solution form had a stronger effect on the proliferation of mouse hybridoma Tg1-1HMS cells than that in the immobilization form [1]. Taken together, the effect of growth factor addition mode on the cells behavior greatly depends on the type of cells. The fourth reason is the type of growth factors used. The activation of bFGF may be different from that of EGF reported previously. [19].

FGF addition into an adipogenic differentiation medium decreased the GPDH activity of fat-derived stem cells and subsequently inhibited adipogenic differentiation [13]. In this study, bFGF in the solution or coated form inhibited the adipogenic differentiation of fat-derived stem cells in terms of mRNA expression of PPARγ2 and aP2, GPDH activity, and the accumulation of oil lipids. bFGF induces the activation of ERK1/2 [15]. It is reported that the ERK1/2 affects the adipogenic differentiation of 3T3-L1 cells [16]. The ERKs inhibit the phosphorylation of PPARγ2 [29, 30]. This indicates that the ERK1/2 activation inhibits the adipogenic differentiation of cells because PPARγ2 is one member of the nuclear receptor superfamilies which contribute to adipogenesis [31]. Taken together, the suppression of adipogenic differentiation would be caused by a decrease in the PPARγ2 mRNA expression which is induced by the ERK1/2 phosphorylation. From the results of GPDH activity and oil lipid production, bFGF in the coated form showed the inhibition effect of adipogenic differentiation similar to that in the solution form (Figures 4 and 5). On the contrary, the inhibition of mRNA expression was stronger for the latter than the former

(Figure 3). The phenomenon may be due to different signaling pathways during the early step of adipogenic differentiation. The second reason may be the denaturation of bFGF coated, as described above. However, at the late stage, the level of differentiation was similar (Figures 4 and 5).

Coating of growth factors is a simple procedure to investigate their effect on cell functions as a juxtacrine fashion. The coating procedure can be used not only for the basic research of cell adhesion and differentiation mechanisms but also for the material design of cell scaffolds to promote their proliferation for tissue regeneration. Growth factors have an affinity for natural substances of collagen and gelatin [32]. Direct coating of growth factors on to a cell scaffold often causes their denaturation, resulting in suppressed biological actions. From the viewpoint of biological activity, the coating with the affinity of nature substances will be promising. Further investigation of the effect of growth factors existence mode on their functions will be needed to control the proliferation and differentiation of stem cells.

REFERENCES

1. G. Chen, Y. Ito and Y. Imanishi *Biochim Biophys Acta* **1358**, 200 (1997)

2. Y. Ito, G. Chen, Y. Imanishi, T. Morooka, E. Nishida, Y. Okabayashi and M. Kasuga *J Biochem (Tokyo)* **129**, 733 (2001)

3. K. Ogiwara, M. Nagaoka, C. S. Cho and T. Akaike *Biotechnol Lett* **27**, 1633 (2005)

4. Y. Naka, A. Kitazawa, Y. Akaishi and N. Shimizu *J Biosci Bioeng* **98**, 348 (2004)

5. Y. Hori, S. Inoue, Y. Hirano and Y. Tabata *Tissue Eng* **10**, 995 (2004)

6. Y. Ito, G. Chen and Y. Imanishi *Biotechnol Prog* **12**, 700 (1996)

7. Y. Ito, J. Zheng, Y. Imanishi, K. Yonezawa and M. Kasuga *Proc Natl Acad Sci U S A* **93**, 3598 (1996)

8. H. Hatakeyama, A. Kikuchi, M. Yamato and T. Okano *Biomaterials* **26**, 5167 (2005)

9. P. A. Zuk, M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz and M. H. Hedrick *Tissue Eng* **7**, 211 (2001)

10. P. A. Zuk, M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim and M. H. Hedrick *Mol Biol Cell* **13**, 4279 (2002)

11. Y. D. Halvorsen, D. Franklin, A. L. Bond, D. C. Hitt, C. Auchter, A. L. Boskey, E. P. Paschalis, W. O. Wilkison and J. M. Gimble *Tissue Eng* **7**, 729 (2001)

12. K. M. Safford, K. C. Hicok, S. D. Safford, Y. D. Halvorsen, W. O. Wilkison, J. M. Gimble and H. E. Rice *Biochem Biophys Res Commun* **294**, 371 (2002)

13. H. Hauner, K. Rohrig and T. Petruschke *Eur J Clin Invest* **25**, 90 (1995)

14. S. Inoue, Y. Hori, Y. Hirano, T. Inamoto and Y. Tabata *J Biomater Sci Polym Ed* **16**, 57 (2005)

15. Y. L. Bao, K. Tsuchida, B. Liu, A. Kurisaki, T. Matsuzaki and H. Sugino *J Endocrinol* **184**, 493 (2005)

16. F. Bost, M. Aouadi, L. Caron and B. Binetruy *Biochimie* **87**, 51 (2005)

17. J. Rao and W. R. Otto *Anal Biochem* **207**, 186 (1992)

18. M. Imamura, A. Kanematsu, S. Yamamoto, Y. Kimura, I. Kanatani, N. Ito, Y. Tabata and O. Ogawa *Am J Physiol Renal Physiol* (2007)

19. A. B. Singh and R. C. Harris *Cell Signal* **17**, 1183 (2005)

20. U. Hersel, C. Dahmen and H. Kessler *Biomaterials* **24**, 4385 (2003)

21. C. J. Marshall *Cell* **80**, 179 (1995)

22. M. Mohammadi, S. K. Olsen and O. A. Ibrahimi *Cytokine Growth Factor Rev* **16**, 107 (2005)

23. D. Moscatelli *J Cell Physiol* **131**, 123 (1987)

24. M. A. Nugent and R. V. Iozzo *Int J Biochem Cell Biol* **32**, 115 (2000)

25. R. Michel, S. Pasche, M. Textor and D. G. Castner *Langmuir* **21**, 12327 (2005)

26. Y. Ito *J Inorg Biochem* **79**, 77 (2000)

27. P. G. Campbell, E. D. Miller, G. W. Fisher, L. M. Walker and L. E. Weiss *Biomaterials* **26**, 6762 (2005)

28. D. R. Newman, C. M. Li, R. Simmons, J. Khosla and P. L. Sannes *Am J Physiol Lung Cell Mol Physiol* **287**, L191 (2004)

29. E. Hu, J. B. Kim, P. Sarraf and B. M. Spiegelman *Science* **274**, 2100 (1996)

30. H. S. Camp and S. R. Tafuri *J Biol Chem* **272**, 10811 (1997)

31. E. D. Rosen and B. M. Spiegelman *Annu Rev Cell Dev Biol* **16**, 145 (2000)

32. Y. Tabata *Tissue Eng* **9 Suppl 1**, S5 (2003)

Chapter 4

Adhesion of human fat-derived stem cells on substrates modified with RGD peptide

INTRODUCTION

Generally, cells adhere on the ECM through an interaction between the ECM component and the corresponding integrin receptor on the cell membrane [1, 2]. A tripeptide motif, RGD, is one of the cell adhesion ligands and is present in ECM proteins, such as fibronection, vitronectin, collagen, and laminin [1]. Many researches have been reported on the cell behavior on culture substrates modified with the RGD [3-7]. It has been demonstrated that the density of RGD immobilized affects the number [5, 6], spreading [6, 7], focal contact formation [4], and migration [4] of cells adhered. The RGD density on the cell substrates can be controlled by changing mixing ratio of monomers with or without a RGD sequence [4, 8]. The density of RGD is also modified by the surface density of functional groups by which RGD is chemically immobilized [5].

Formation technique of SAM with alkanethiols and silanes has been used for the surface modification of cell culture substrates, because by the SAM technique it is easy to change the density of functional groups on the substrate surface [9-11]. A RGD peptide or RGD-containing proteins have been immobilized through the functional groups of SAM surface [12-14]. It is known that the chemical component of SAM is easily exchanged by a simple immersion in ethanol solution containing an alkanethiol [15, 16]. The exchange rate of SAM component can be changed by the time period of immersion. This procedure will be effective in changing the chemical composition of SAM surface, resulting in modified surface density of bioactive peptides and proteins immobilized. However, no research has been reported on the modification of surface density by the SAM exchange technique.

This Chapter investigates the adhesion behavior of human fat-derived stem cells on the RGD-immobilized SAM. The SAM of methyl groups was immersed in ethanol solution containing an alkanethiol with one carboxyl group for different time periods and exchanged to the SAM of carboxyl groups at different surface densities. RGD was immobilized to the carboxyl groups of SAM prepared to obtain the SAM with different densities or gradients of RGD immobilized. The surface properties of SAM were characterized by the water contact angle and X-ray photoelectron spectroscopy (XPS) measurements. Fat-derived stem cells isolated from human adipose tissue were seeded on the RGD-immobilized SAM and the number, morphology, and alive percentage of cells

adhered were evaluated in terms of the RGD density.

EXPERIMENTAL

Preparation of substrates modified by a SAM exchange technique

1-undecanethiol, $HS(CH_2)_{10}CH_3$ (CH_3), and 2-(2-(2-(2-(2-(2-(2-(11-mercaptoundecyloxy)-ethoxy)ethoxy)ethoxy)ethoxy)ethoxy)ethoxy)acetic acid, $HS(CH_2)_{11}(OCH_2CH_2)_6OCH_2COOH$ (COOH), were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and Toyobo Co. Ltd. (Osaka, Japan), respectively. Two types of RGD-immobilized SAM were prepared by the SAM exchange technique. One is a COOH-exchanged SAM which has a uniform density of carboxyl groups on the surface (Figure 1 (A)). The other is a COOH-gradient SAM which has a gradient density of carboxyl groups on the surface (Figure 1 (B)). PET film was kindly supplied by Teijin Ltd. (Tokyo, Japan) and washed for 15 min with methanol by an ultrasound bath (Brason 2510, Yamato Tokyo, Japan), followed by rising with DDW. The PET film was punched out into a round shape of 14 mm in diameter and cut into the square shape of 10×40 mm^2 for SAM preparation. A 50 nm gold layer was deposited on the surface of round and square PET films at Muranaka Medical Instrument Co., Ltd. (Osaka, Japan). The gold-deposited films were immersed for 24 hr in 1 mM ethanol solution of CH_3 and COOH to prepare CH_3 and COOH SAM, respectively. [15, 16] The SAM prepared were thoroughly rinsed with ethanol to exclude alkanethiols unreacted and dried. To prepare COOH-exchanged SAM, the CH_3 SAM were immersed for 6, 11, 18, and 24 hr in 1 mM ethanol solution of COOH to allow to partially exchange to carboxyl groups (Figure 1 (A)). To prepare COOH-gradient SAM, the CH_3 SAM were immersed for 6 hr in 1 mM ethanol solution of COOH to allow to paratially exchange to carboxyl groups (Figure 1 (B)). The two types of CH_3 and partially exchanged SAM were fixed on a slide glass and set to a COOH gradient maker where COOH was added in a certain volume by a syringe pump (STC-525, Terumo Co., Tokyo, Japan). The solution surface constantly raises at a rate of 5 mm/min and the film was immersed in the COOH solution for different time periods to generate COOH-gradient SAM on it.

Characterization of SAM-modified substrates

The surface of SAM was characterized by the water contact angle and XPS measurements. The water contact angle was measured with a geometer (Contact angle meter CA-X, Kyowa Interface Science, Saitama, Japan) according to the method previously reported [17]. Briefly, water (9 μl) was dropped onto the surface of SAM at room temperature while at the same time point after water dropping, the water contact angle was measured independently 15 times for

SAM. The XPS measurement was performed by an Electron Spectroscopy for Chemical Analysis (ESCA-850V, Shimazu Co., Kyoto, Japan) at 8 kV and 30 mA below 1×10^{-6} torr and the data were analyzed by Vision-330 (Shimazu Co.) [18].
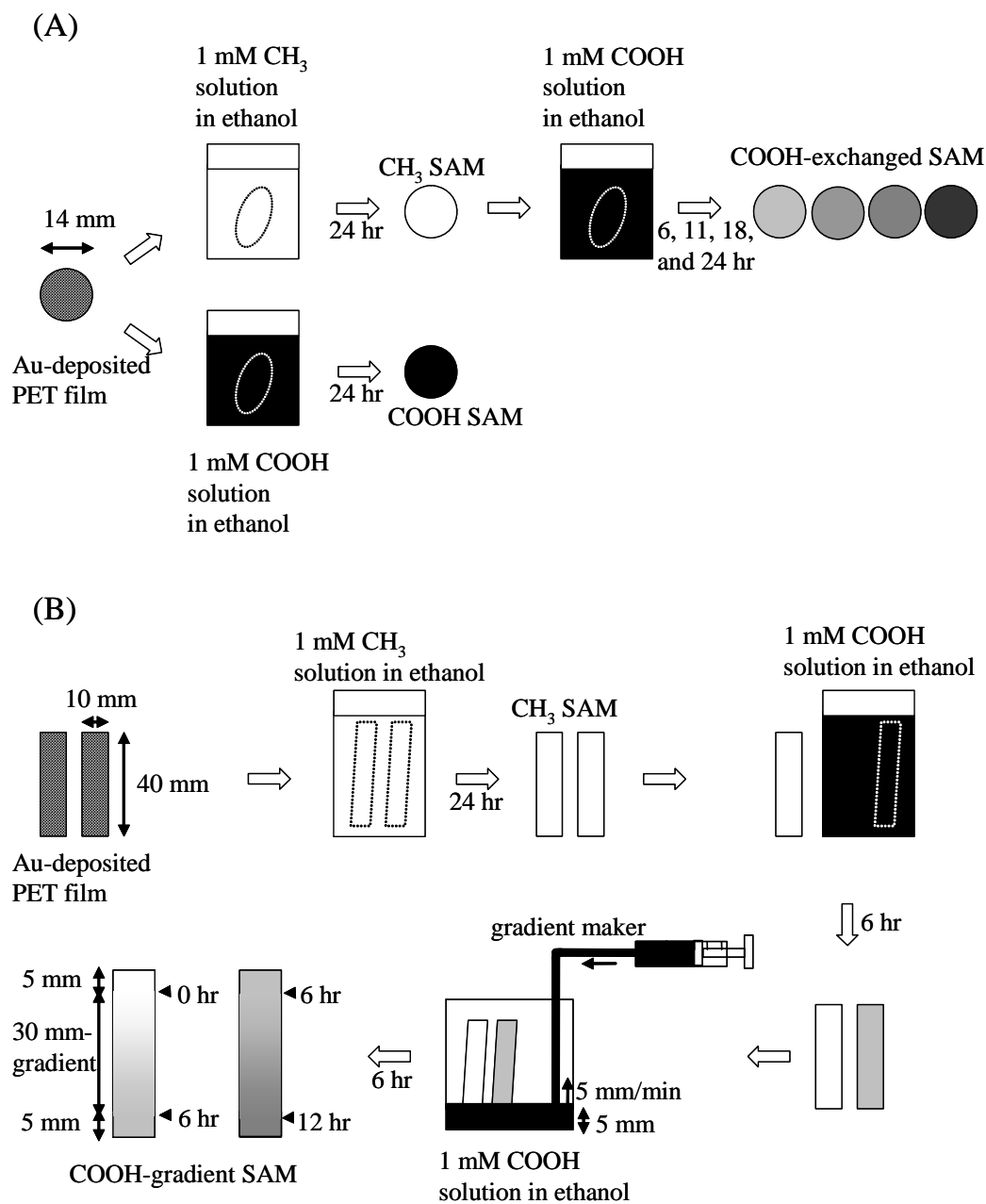

Figure 1. Schematic illustration to prepare substrates with different surface densities (A) and gradients of COOH groups (B).

Immobilization of RGD on SAM-modified substrates

RGD peptides were prepared by the conventional peptide synthesis method and kindly supplied from Department of Applied Chemistry, Osaka Institute of Technology (Osaka, Japan).

The RGD peptide (GGGRGDSP) was chemically immobilized through the carboxyl groups of SAM. The SAM films were sterilized in 70% aqueous solution of ethanol and washed with sterilized phosphate-buffered saline solution (100 mM, pH 7.4, PBS). Then, they were placed in sterilized PBS solution (250 μl/cm^2) containing 0.2 M of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.05 M of N-hydroxysuccinimide (NHS) for 20 min to pretreat for the activation of carboxyl groups [19, 20]. After washing with sterilized PBS, the SAM films were placed in sterilized PBS solution (250 μl/cm^2) containing 20 μg/ml of RGD peptide for 2 hr at 37°C. The SAM films were washed with PBS to prepare SAM films with different densities and gradients of RGD immobilized. As controls, the SAM film was placed in the RGD solution without EDC/NHS pretreatment.

Measurement of density of RGD immobilized

The amount of RGD peptide immobilized was measured by a radiolabeling method. The RGD peptide (GGGRGDSY) was radioiodinated according to the conventional chloramine-T method [21]. Briefly, 10 μl of Na^{125}I (PerkinElmer Japan Co. Ltd., Kanagawa, Japan) was added to 400 μl of 1 mg/ml RGD solution in 0.5 M potassium phosphate-buffered solution (pH 7.5) containing 0.5 M sodium chloride (reaction buffer). Then, 200 μl of the reaction buffer containing 0.2 mg/ml of chloramine-T was added to the solution mixture. After agitating at room temperature for 2 min, 200 μl of PBS containing 4mg/ml of sodium metabisufate was added to the solution mixture to stop the raioiodination. The reaction mixture was passed through a column (Dowex I-X8, Muromachi Technology Co. Ltd., Tokyo, Japan) to remove the uncoupled ^{125}I molecule from the ^{125}I-labeled RGD. ^{125}I-labeled RGD was used for immobilization on SAM after the concentration was determined by Micro BCA protein assay kit (#HJ106352, Pierce, Thermo Fisher Scientific Inc., IL). The amount of RGD immobilized on SAM was measured by a γ counter (Auto Well Gamma System, Aloka, Tokyo, Japan).

Isolation and culture of human fat-derived stem cells

Fat-derived stem cells were primarily isolated from the human fat tissue that had been collected in the reduction mammaplastic surgery of breast cancer patients receiving the informed consent at Kyoto University Hospital. The fat tissue (5 ml) after removing cancer and fibrous tissues was washed with PBS to carefully remove blood cells, then minced, and digested by 520 U/ml collagenase (Nitta Gelatin Inc., Osaka, Japan) at 37 ^{o}C for 15 min under shaking [22]. The digested was suspended in Medium 199 (Sigma-Aldrich Co., MO) containing 10 vol% fetal calf serum (FCS, #AJL12371, Hyclone Laboratories Inc., UT). The suspension was filtered through a nylon mesh with 200 μm opening and centrifuged at 1,000 rpm for 5 min at 4 ^{o}C. After washing

twice with the culture medium, the cells obtained were suspended in the culture medium and cultured at 37 ^{o}C in a 75-cm^2 culture flask (#430720, Corning Inc., NY). Non-adherent cells were removed 1 day later and adherent cells were proliferated in the culture medium containing 1 μg/ml of basic fibroblast growth factor (bFGF, kindly supplied from Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) [22]. After incubation for 7 days, the cells in near confluence conditions were subcultured, followed by further incubation for proliferation.

Cell culture on RGD-immobilized substrates

Fat-derived stem cells cultured were detached with PBS containing 0.25 wt% trypsin and 0.2 wt% ethylenediamineteraacetic acid. Then, trypsin inhibitor (1 mg/ml) in Medium 199 was added to inhibit the trypsin activity [23]. After centrifugation at 1,000 rpm for 5 min at 4 ^{o}C, cells were suspended into Medium 199 containing ITS solution (mixture solution of insulin (10 μg/ml), transferrin (5.5 μg/ml), and sodium selenite (5 ng/ml), Sigma-Aldrich Co.). The cell suspension solution was placed onto the COOH-exchanged and COOH-gradient SAM with RGD immobilization at a density of 1×10^4 cells/cm^2.

Measurement of cell number

The number of cells adhered after 1 hr incubation was measured by a DNA assay method [24]. Briefly, fat-derived stem cells were rinsed twice with PBS, underwent a freeze and thaw process, and finally incubated in 1 ml of aqueous solution containing 0.2 mg/ml sodium dodecyl sulfate, 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate for 1 hr for cell lysis. The cell lysate (100 μl) was mixed with 100 μl solution of 1 μl/ml Hoechst 33258 dye solution (bisbenzimide H33258 fluorochrome trihydrochloride DMSO solution, #L2E4809, Nacalai Tesque, Inc., Kyoto, Japan), 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate, and then the fluorescence intensity of mixed solution was measured on a fluorescence spectrophotometer (Spectra Max Gemini Em, Molecular Device Japan Co., Osaka, Japan) at exciting (Ex) and emission (Em) wavelengths of 355 and 460 nm. The cell number was determined by use of the calibration curve prepared by the cells of known number.

Measurement of cell spreading area

The morphology of fat-derived stem cells adhered was observed by a phase-contrast microscopy (IX70, Olympus Optical Co., Ltd., Tokyo, Japan) after 24 hr cell culture. The cell-spreading area was measured by a computer program of MetaMorph (Molecular Devices Inc., Ontario, Canada). For every SAM surface, the spreading area of 100 cells was measured.

Measurement of alive/dead cell number

The cells adhered were stained by a alive/dead viability cytotoxicity assay kit (#42830A, Molecular Probes, Invitrogen Co., CA) to assess live and dead cells [25]. The cells incubated for 24 hr were washed with PBS fifth times and incubated with the kit solution containing fluorescence-labeled calcein (Ex:495 nm, Em:515 nm) and ethidium homodimer (Ex:495 nm, Em:635 nm) for 5 min. After washing with PBS fifth times, the stained cells were observed by confocal laser scanning microscope (Olympus Fluroview FV300 confocal laser scanning microscope, Olympus Optical Co.). The number of cells stained in green color (live) or red color (dead) was counted to calculate the percentage of alive cells of 100 cells per every SAM sample.

Data analysis

All the data were expressed as the mean $\pm$ the standard deviation of the mean. The data of cell culture were statistically analyzed by the analysis of variance (ANOVA) with post-hoc Fisher's PLSD test. Statistical significance was accepted at $p < 0.05$.

RESULTS

Characterization of CH_3, COOH, and CH_3/COOH SAM

Figures 2 (A) and (B) show the water contact angle and surface elementary analysis data of SAM. The water contact angle of SAM decreased with an increase in the time period of immersion in the COOH ethanol solution. The molecular percentage of carbon and oxygen decreased and increased with an increase in the immersion period, respectively. It is apparent that the CH_3 groups were exchanged by the COOH groups. The CH_3/COOH ratio could be changed with time. The molecular percentage of oxygen for SAM prepared by immersion for 18 and 24 hr was as same as that of COOH SAM. However, the water contact angle of the former was different from that of the later.

Density of RGD immobilized on SAM-modified substrates

Figure 3 shows the density of RGD on SAM. For the COOH-exchanged SAM pretreated with EDC and NHS, the density of RGD increased with an increase in the time period of immersion in the COOH solution. However, without EDC and NHS pretreatment, RGD density was constant and lower than that of pretreated SAM. No influence of EDC and NHS pretreatment on the RGD density was observed for CH_3 SAM. The RGD density of COOH SAM was similar, irrespective of the EDC and NHS pretreatment.
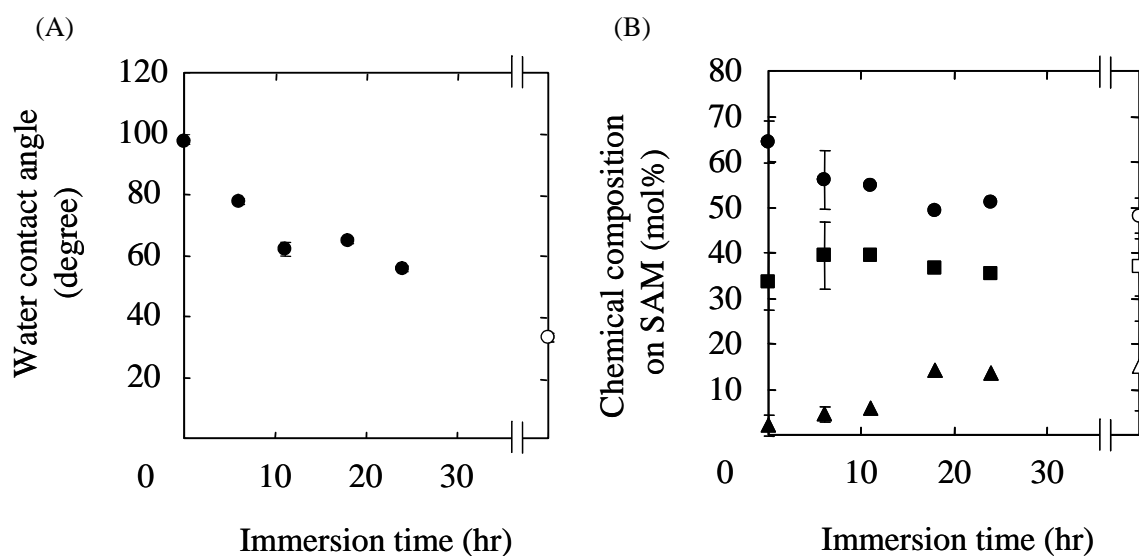
- 70 -

(A)

(B)

Figure 2. (A) Water contact angle of CH$_3$ SAM surfaces treated with the ethanol solution of COOH for different immersion time periods (●). As a control, the water contact angle of the COOH SAM surface was measured (○). (B) Chemical compositions of C (● and ○), O (▲ and △), and Au (■ and □) for CH$_3$ SAM surfaces treated with the ethanol solution of COOH for different immersion time periods (●, ▲, and ■). Open marks (○, △, and □) indicate the chemical composition of COOH SAM surface.
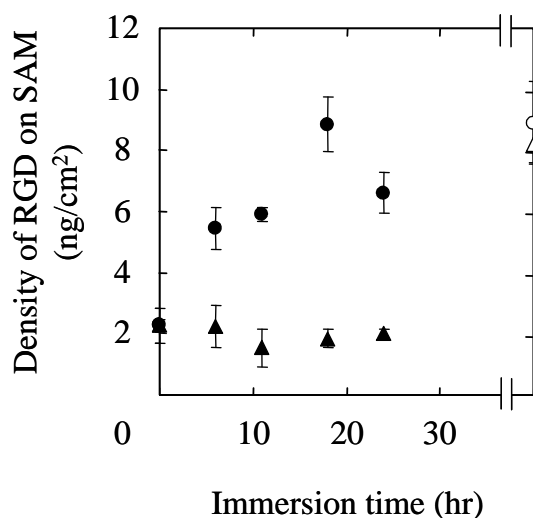

Figure 3. Density of RGD on CH$_3$ SAM surfaces treated with the ethanol solution of COOH for different immersion time periods (● and ▲). RGD immobilization was performed for 2 hr following 20 min pretreatment with (● and ○) or without EDC and NHS (▲ and △). Open marks (○ and △) indicate the density of RGD on the COOH SAM surface.

Number of cells adhered on RGD-immobilized substrates

Figure 4 shows the number of fat-derived stem cells incubated for 1 hr on different densities of RGD immobilized on CH_3, COOH, COOH-exchanged SAM. No significant difference in the cell number was observed in terms of RGD density.
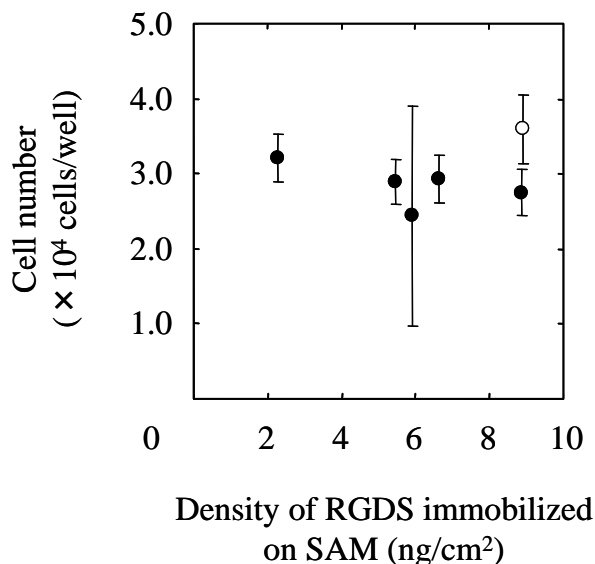

Figure 4. Number of fat-derived stem cells attached after 1 hr incubation onto the CH_3 SAM surfaces treated with the ethanol solution of COOH for different immersion time periods (●). The open mark (○) indicates the number of cells onto the COOH SAM surface. RGD immobilization was performed with the EDC and NHS pretreatment.

Cell spreading on RGD-immobilized substrates

Figure 5 shows the phase-contrast micrographs of fat-derived stem cells adhered onto SAM after 24 hr culture. For the COOH SAM reacted in RGD solution after pretreatment with EDC and NHS, fat-derived stem cells spread on it. However, the cells showed a round shape on the COOH SAM reacted in RGD solution without EDC and NHS pretreatment. On the COOH SAM and CH_3 SAM reacted in RGD solution after EDC and NHS pretreatment, the cell morphology was round.

Figure 6 (A) shows the phase-contrast micrographs of fat-derived stem cells adhered on different densities of RGD immobilized on CH_3, COOH, and COOH-exchanged SAM after 24 hr culture. The morphology of cells adhered depended on the immersion time period. The cells on the CH_3 SAM showed a round shape, whereas they spread on the COOH-exchanged SAM. Figure 6 (B) shows the cell spreading area of fat-derived stem cells incubated for 24 hr on CH_3, COOH, and COOH-exchanged SAM. The cell spreading area increased with an increase in the density of RGD. The cell spreading area on the COOH SAM was similar to that of the CH_3 SAM, but smaller than that of COOH-exchanged SAM.
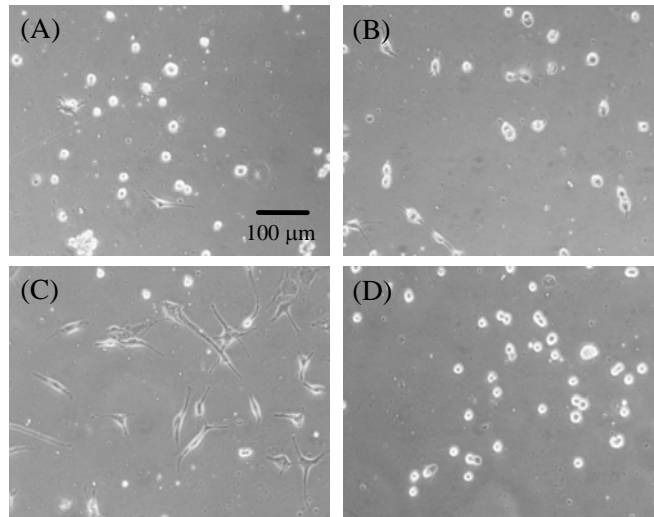
Figure 5. Phase-contrast microscopic photographs of fat-derived stem cells attached for 24 hr onto the COOH (A, B, and C) and CH_3 SAM (D). RGD immobilization was not performed (A) and was done with (C and D) or without the pretreatment of EDC and NHS (B).
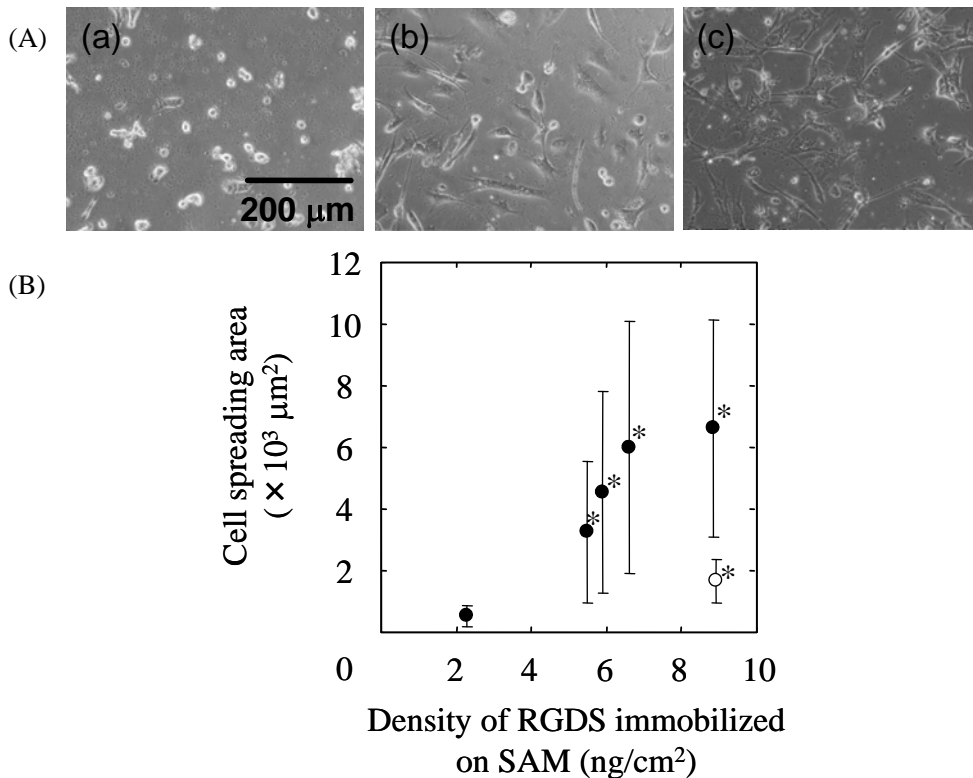

Figure 6. (A) Phase-contrast microscopic photographs of fat-derived stem cells attached for 24 hr onto the CH_3 SAM surfaces treated with the ethanol solution of COOH for 0 (a), 11 (b), and 24 hr (c). (B) Spreading areas of fat-derived stem cells after 24 hr incubation onto the CH_3 SAM surfaces treated with the ethanol solution of COOH for different time periods (closed marks). The open mark indicates the spreading area of cells onto the COOH SAM surface.
*, $p < 0.05$; significant against the cell spreading area for the CH_3 SAM prepared by 0 hr treatment.

Alive/dead cell number on RGD-immobilized substrates

Figure 7 (A) shows the confocal laser scanning micrographs of fat-derived stem cells stained to evaluate their live/dead viability. Most of cells were dead on the CH_3 SAM, but the cells on the COOH-exchanged SAM were alive. Figure 7 (B) shows the percentage of alive cells after 24 hr incubation on CH_3, COOH, and COOH-exchanged SAM with different densities of RGD immobilized. The percentage of alive cells increased when the density of RGD became high.
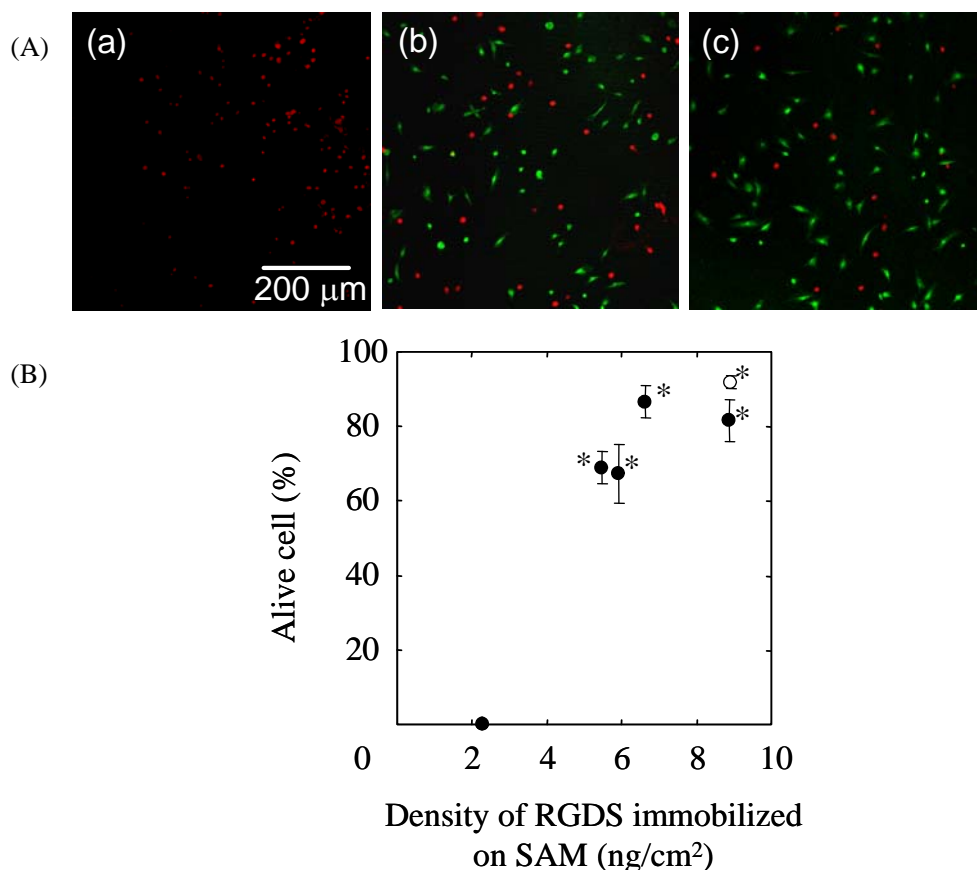

Figure 7. (A) Confocal laser scanning images of alive/dead assay for fat-derived stem cells after 24 hr incubation onto the CH_3 SAM surfaces treated with the ethanol solution of COOH for 0 (a), 11 (b), and 24 hr (c). (B) Percentage of alive fat-derived stem cells after 24 hr incubation onto the CH_3 SAM surfaces treated with the ethanol solution of COOH for different time periods (●). The open mark (○) indicates the percentage of alive cells onto the COOH SAM surface.
*, $p<0.05$; significant against the percentage of alive cells for the CH_3 SAM prepared by 0 hr treatment.

Figure 8 shows the percentage of alive cells incubated for 24 hr on different gradients of RGD immobilized on COOH-gradient SAM. The distance from one end of gradients depended on the immersion time period in COOH solution (Figure 1 (B)). The gradients were prepared during the length of 30 mm on 40 mm-PET films. The percentage of alive cells was evaluated at 5, 10, 15,

20, and 25 mm from one end of the COOH-gradient SAM prepared for 0-6 and 6-12 hr immersion periods to avoid a side effect of the gradient. On the gradient SAM prepared for 6-12 hr immersion period, the percentage of alive cells increased with an increase in the time period of immersion in the COOH solution. However, no effect of immersion time period on the percent cell alive was observed for the 0-6 hr gradient SAM.
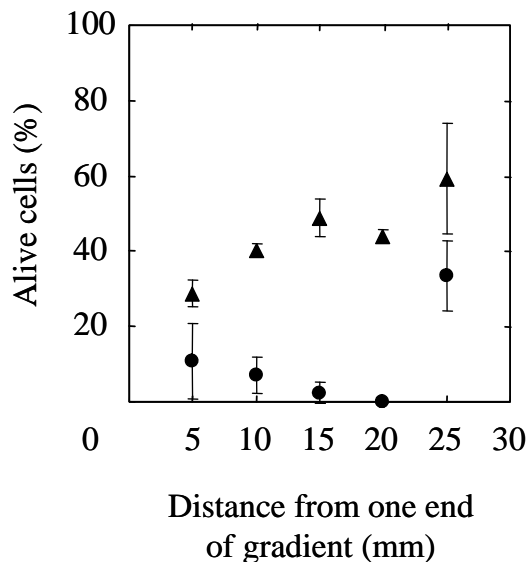

Figure 8. Percentage of alive fat-derived stem cells after 24 hr incubation onto the CH_3 SAM surfaces treated with the ethanol solution of COOH for 0-6 (●) and 6-12 hr (▲). The 30 mm-COOH-gradient SAM were prepared on 40 mm-PET films according the procedure described in Figure 1 (B). The percentage of alive cells was evaluated at 5, 10, 15, 20, and 25 mm from one end of the gradient. The site points correspond to the COOH-gradient SAM prepared at the immersion time periods of 1, 2, 3, 4, and 5 hr (●) or 7, 8, 9, 10, and 11 hr (▲), respectively.

DISCUSSION

The RGD density of substrates affects the behavior of cell adhesion on them, such as the number [5, 6], spreading [6, 7], focal contact formation [4], and migration [4] of cells. In this study, the density and gradient of RGD immobilized could be changed by the density and gradient of carboxyl groups which can be regulated by the SAM exchange technique [15, 16]. The number and spreading of cells adhered were modified by the different densities and gradients of RGD. In addition, RGD immobilization affected the percent alive of cells.

SAM with gradient of functional groups have been prepared by the mixed SAM technique. The SAM surface with two types of functional groups can be formed by simply mixing the two types of alkanethiols in ethanol solution [18]. The surface composition on the mixed SAM

is governed by the chemical composition of alkanethiols used. When the two kinds of alkanethiol solution are diffused from opposite sides in a gel, the concentration gradient of alkanethiols is formed in the gel by cross-diffusion of alkanethiols [26]. Smith et al. formed the gradient SAM by the SAM mix technique and immobilized fibronectin on the gradient SAM. The fibronectin-graident SAM affected cell migration [20]. In this paper, the SAM exchange technique was used to change the surface density of functional groups and form the surface gradient. This technique is more controllable than that of SAM mix technique, because the slop of gradient can be artificially regulated by changing the addition volume of alkanethiol solution with a syringe pump.

CH$_3$, COOH, and COOH-exchanged SAM were evaluated by the water contact angle and XPS measurements [27]. The water contact angle and the oxygen composition of the COOH-exchanged SAM decreased and increased with an increase in the time period of immersion in the COOH ethanol solution, respectively. However, the extent changed was different (Figures 2 (A) and (B)). In general, the number of functional groups immobilized onto a surface is not proportional to the water contact angle of the surface [28]. The water contact angle of COOH-exchanged SAM immersed for 18 and 24 hr reached a certain saturated level which is different from that of COOH SAM (Figure 2 (A)). Since the molecular length of COOH, -(OCH$_2$CH$_2$)$_6$-OCH$_2$COOH, is longer than that of CH$_3$, it is possible that the SAM surface was almost covered with the COOH although the COOH coupling ratio was low. This may result in the similar chemical composition measured from the XPS. However, the COOH density of COOH-exchanged SAM is lower than that of COOH SAM. Consequently, the water contact angle of COOH-exchanged SAM would be higher than that of COOH SAM. The flexibility of SAM also affects the free motion of RGD molecules. It is highly conceivable that the higher free motion of RGD molecules, the more frequently the RGD can be recognized by the surface receptor of cells. When compared between the COOH and COOH-exchanged SAM, the COOH flexibility of the latter would be higher, resulting in the higher susceptibility to receptor recognition. This may cause higher spreading area of cells. The RGD density of SAM surface pretreated with EDC/NHS was higher for the COOH SAM than the COOH-exchanged SAM. This is due to the higher COOH density.

RGD has been immobilized on the surface of cells substrates by using various coupling reagents [3]. Selection of functional groups for the RGD coupling is important, because some groups often induce the spontaneous adsorption of protein and peptide which affects the adhesion, spreading, proliferation, and differentiation of cells [11, 29, 30]. As apparent from Figure 3, the CH$_3$ SAM showed the RGD density around 2 ng/cm^3, but the density did not increased by the RGD coupling reaction with the EDC/NHS pretreatment. This is because the CH$_3$ SAM do not have reactive groups. The RGD on the CH$_3$ SAM had no effect on cell spreading (Figure 5 (d)). This

indicates that RGD physically adsorbed on the CH_3 SAM did not affect the cell adhesion. The density of RGD on COOH-exchanged SAM increased with an increase in the time period of immersion after EDC and NHS pretreatment. The density of RGD immobilized on COOH-exchanged SAM with pretreatment was higher than that without pretreatment. This indicates the chemical immobilization of RGD onto the COOH-exchanged SAM. The RGD density on the COOH SAM was same, irrespective of the EDC and NHS pretreatment. This is because RGD was physically adsorbed on the COOH SAM. The length and flexibility of COOH molecules may generate a space between the COOH molecules immobilized onto COOH SAM, resulting in the sorption of RGD between the COOH molecules or the adsorption onto the gold-coated surface directly. In Figure 4, the RGD immobilized or absorbed on the COOH SAM showed the similar number of cells adhered. However, the spreading of cells was quite different to each other (Figure 6). It is highly conceivable that the RGD molecules sorbed or adsorbed were not biologically recognized by cells even though the amount of RGD surface modified was similar. As a result, the cell spreading would be not induced. For the physical sorption and adsorption of RGD molecules, it is likely that the molecules are covered by COOH chains and directly place on the substrate surface not to move freely to be recognized by the corresponding receptor. However, the reason why the biological recognition is different between the RGD molecules sorbed or adsorbed and immobilized is not clear.

RGD was immobilized on the exchanged SAM. The highest density was 8.9 ng/cm^2. The density of RGD peptide was 7.0×10^{13} peptides/cm^2 on the assumption of the molecular size and monolayer immobilization. It is reported that the distance between alkanethiols is 4.6-4.7 Å [9]. Considering that the density of alkanethiols is 4.8×10^{14} alkanethiols/cm^2, one RGD molecule was immobilized on one out of 6.8 COOH groups. Cell adhesion behavior is greatly influenced by the density of RGD. Yoon et al showed that the density of 2-8 pmol/cm^2 affected cell adhesion [5]. Maheshwan et al. showed that the density of 0.17-17 pmol/cm^2 affected cell migration, adhesion strength, and focal contact formation [4]. In this paper, the density of 3.0-12 pmol/cm^2 (2.3-8.9 ng/cm^2) was immobilized on the exchanged SAM. The similar effect of RGD density was observed for fat-derived stem cells.

When cells adhere on the substrate mainly by the interaction between the integrin on the cell membrane and RGD molecules on the ECM, they begin to flatten and their plasma membrane spread, leading to the actin organization and the occurrence of focal adhesion [1]. The phenomenon is induced by integrin signals [2]. The integrin signals also control migration, proliferation, and survival of cells [2]. Our results indicate the density of RGD depended on the spreading and alive percentage of fat-derived stem cells. It is possible that the activation of integrin signal pathway by the interaction of RGD and integrin dependeds on the RGD density, which affects the downstream

signals for spreading and survival of cells [8]. Generally, when cells detach from the ECM, the integrin signals are shut down. The suppression of integrin signals induces anoikis which is one of the apoptosis [31]. Cell death was observed on the CH_3 SAM (Figure 7 (A)). The cell death might be induced by the suppression of integrin signals. Distinct from the cell spreading, the similar alive percentage of cells was observed at the RGD density of 2 ng/cm^2 for both of the SAM surface sorbed and adsorved and chemically immobilized by RGD. It is possible that the RGD molecules stimulate the integrin receptor, resulting in induction of intercellular signals to suppress cell death. On the COOH-exchanged SAM, the mixture of dead and alive cells was observed. It has been reported that the islands of alkanethiol (-10 nm^2) are formed on the SAM surface [32]. The islands of CH_3 cannot immobilize chemically RGD molecules. On the other hand, the size of fat-derived stem cells was 400-9500 μm^2. The cells are too large to biologically recognize the island formation of CH_3 SAM without RGD immobilization. The cell death is not caused not by the island formation of CH_3 SAM, but by the inherent nature of adipo-stomal cells. The adipo-stomal cells may be sensitive to the conditions of adhesion substrates.

The effect of RGD density on the cell survival was evaluated by using the COOH-exchanged and COOH-gradient SAM. The surface property at 5 mm for the COOH-gradient SAM of 0-6 hr immersion corresponds to that for COOH-exchanged SAM of 1 hr immersion, which is most similar to the CH_3 SAM with the RGD density of 2 ng/cm^2. The surface properties at 25 mm for the COOH-gradient SAM of 0-6 hr immersion and at 5 mm for the COOH-gradient SAM of 6-12 hr immersion correspond to those of the COOH-exchanged SAM immersed for 5 and 7 hr immersion, respectively, which are almost similar to the COOH-exchanged SAM of 6 hr immersion with the RGD density of 5.4 ng/cm^2. The surface property at 25 mm for COOH-gradient SAM of 6-12 hr immersion corresponds to that of the COOH-exchanged SAM of 11 hr immersion with the RGD density of 5.9 ng/cm^2. Percentage of alive cells at these positions increased with an increase in total immersion time. The RGD effect of COOH-gradient SAM on the percentage of alive cells seemed to be in accordance with that of COOH-exchanged SAM. If the CH_3 SAM are immersed for the same time period in the COOH solution, it is highly conceivable that the surface density of carboxyl groups is almost identical for both preparation procedures of COOH-exchanged and COOH-gradient SAM. After that, since the RGD immobilization was performed in the similar manner, it is possible that both the SAM surfaces have the similar density of RGD immobilized. Generally, cell behavior on gradient surface is influenced not only by the local environment, but also by the culture medium around cells [20]. The cells adhered on locally different environments may secrete proteins or generate cellular signals in different concentrations and manners. However, from the present finding, there was not big influence of the secreted on the cell behavior. The cell survival on the COOH-gradient SAM immersed for 0-6 hr did not depend on

the total immersion time. The density on the COOH-gradient SAM might have a threshold to affect the cell behavior.

Fat-derived stem cells which can be isolated from the adipose tissue, are capable of differentiating into adpogenic, osteogenic, chondrogenic, neurogenic, and myogenic cells [33, 34]. The spreading and survival of the fat-derived stem cells could be controlled by the density and gradient of RGD immobilized on COOH-exchanged and COOH-gradient SAM. When proteins, such as growth factors which affect the proliferation and differentiation of cells, are immobilized on the surface, the proliferation and differentiation of cells will be controllable by the change of density and gradient.

REFERENCES

1. U. Hersel, C. Dahmen and H. Kessler *Biomaterials* **24**, 4385 (2003)

2. W. Guo and F. G. Giancotti *Nat Rev Mol Cell Biol* **5**, 816 (2004)

3. K. H. Park, K. Na and H. M. Chung *Biotechnol Lett* **27**, 227 (2005)

4. G. Maheshwari, G. Brown, D. A. Lauffenburger, A. Wells and L. G. Griffith *J Cell Sci* **113 (Pt 10)**, 1677 (2000)

5. J. J. Yoon, S. H. Song, D. S. Lee and T. G. Park *Biomaterials* **25**, 5613 (2004)

6. M. Schuler, G. R. Owen, D. W. Hamilton, M. de Wild, M. Textor, D. M. Brunette and S. G. Tosatti *Biomaterials* **27**, 4003 (2006)

7. D. L. Hern and J. A. Hubbell *J Biomed Mater Res* **39**, 266 (1998)

8. W. A. Comisar, N. H. Kazmers, D. J. Mooney and J. J. Linderman *Biomaterials* **28**, 4409 (2007)

9. A. Ulman *Chem Rev* **96**, 1533 (1996)

10. C. D. Bain, J. Evall and G. M. Whitesides *J Am Chem Soc* **111**, 7155 (1989)

11. B. G. Keselowsky, D. M. Collard and A. J. Garcia *J Biomed Mater Res A* **66**, 247 (2003)

12. B. T. Houseman and M. Mrksich *Biomaterials* **22**, 943 (2001)

13. M. Kato and M. Mrksich *Biochemistry* **43**, 2699 (2004)

14. J. Lahiri, L. Isaacs, J. Tien and G. M. Whitesides *Anal Chem* **71**, 777 (1999)

15. H. A. Biebuyck and G. M. Whitesides *Langmuir* **9**, 1766 (1993)

16. D. M. Collard and M. A. Fox *Langmuir* **7**, 1192 (1991)

17. Y. Hori, S. Inoue, Y. Hirano and Y. Tabata *Tissue Eng* **10**, 995 (2004)

18. C. D. Bain, J. Evall and G. M. Whitesides *J Am Chem Soc* **111**, 7155 (1989)

19. N. Nakajima and Y. Ikada *Bioconjug Chem* **6**, 123 (1995)

20. J. T. Smith, J. K. Tomfohr, M. C. Wells, T. P. Beebe, Jr., T. B. Kepler and W. M. Reichert *Langmuir* **20**, 8279 (2004)

21. Y. Kimura, M. Ozeki, T. Inamoto and Y. Tabata *Tissue Eng* **8**, 603 (2002)

22. S. Inoue, Y. Hori, Y. Hirano, T. Inamoto and Y. Tabata *J Biomater Sci Polym Ed* **16**, 57 (2005)

23. Y. L. Bao, K. Tsuchida, B. Liu, A. Kurisaki, T. Matsuzaki and H. Sugino *J Endocrinol* **184**, 493 (2005)

24. J. Rao and W. R. Otto *Anal Biochem* **207**, 186 (1992)

25. R. M. Coleman, N. D. Case and R. E. Guldberg *Biomaterials* **28**, 2077 (2007)

26. B. Liedberg and P. Tengvall *Langmuir* **11**, 3821 (1995)

27. Y. Xia and G. M. Whitesides *Angew Chem Int Ed* **37**, 550 (1998)

28. Y. Ikada *Biomaterials* **15**, 725 (1994)

29.   N. Faucheux, R. Schweiss, K. Lutzow, C. Werner and T. Groth *Biomaterials* **25**, 2721 (2004)

30.   J. M. Curran, R. Chen and J. A. Hunt *Biomaterials* **26**, 7057 (2005)

31.   P. J. Reddig and R. L. Juliano *Cancer Metastasis Rev* **24**, 425 (2005)

32.   G. E. Poirier *Chem Rev* **97**, 1117 (1997)

33.   P. A. Zuk, M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz and M. H. Hedrick *Tissue Eng* **7**, 211 (2001)

34.   P. A. Zuk, M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim and M. H. Hedrick *Mol Biol Cell* **13**, 4279 (2002)

PART II

CULTURE OF HUMAN FAT-DERIVED STEM CELLS ON

THREE-DIMENSIONAL SUBSTRATES WITH

DIFFERENT SURFACE PROPERTIES

Chapter 5

Proliferation and osteogenic differentiation of human fat-derived stem cells on PET non-woven fabrics by different culture methods

INTRODUCTION

It is important for tissue engineering to efficiently prepare the cells of high proliferation and differentiation potentials. To this end, one standard strategy is to isolate such cells from the body tissue, and then to expand them by a cell culture method. It is well recognized that cells basically proliferate and function in the body interacting with the ECM which is the nature scaffold of cells [1, 2]. Taking this *in vivo* system into consideration, it is conceivable effective proliferation of cells cannot be always expected without any cell scaffolds. Thus, cells should be seeded into a suitable scaffold and the cell-scaffold construct should be cultured under appropriate conditions for successful cell proliferation. Some researches have demonstrated that the scaffold is important to physically and biologically support the adhesion, proliferation and differentiation of cells [3-7]. Moreover, the homogeneous distribution of cells throughout the scaffold is also one of the key factors contributing to cell proliferation [8].

MSC isolatable from the bone marrow have been attracted great attention for their applications to tissue engineering since they have an inherent potential to differentiate into adipocytes [9, 10], chondrocyte [10, 11], myoblasts [12, 13], and osteoblasts [10, 11]. Recent researches have demonstrated that there is a population of multipotent stromal cells in the extramedullary fat tissue which have the similar differentiation potential to MSC [14, 15]. Compared with the isolation procedure of MSC by the spinal punctuation, it is easy and less painful for patients to obtain fat-derived stem cells from fat tissues. However, little has been investigated on the proliferation and differentiation behaviors of cells in the 3-demensional scaffold from the viewpoint of their tissue engineering application, although some biological researches of cells have been performed [14-16].

The objective of this study is to obtain fundamental knowledge about the behavior of fat-derived stem cells cultured in the non-woven fabrics of cell scaffold. The stromal cells were isolated from the human fat tissue and their proliferation and differentiation profiles were investigated in the non-woven fabrics prepared from PET fiber with different diameters which have various porosities. The cells were cultured by a static, stirred, and agitated method to evaluate the effect of culture method on the proliferation and osteogenic differentiation of cells in the

non-woven fabrics.

EXPERIMENTAL

Preparation of PET non-woven fabrics

Non-woven fabrics prepared by a melt-blow method from the PET fiber with different diameters were kindly supplied from Toray Co., Shiga, Japan. The non-woven fabrics were further processed either by thermal compression or disentanglement of fibers to make their porosity uniform. Briefly, the fabrics were punched out into 6 mm-diameter disks. The fabric disks were compressed with two glass plates at 150℃ for 30 min to adjust the porosity at 94 % (low porosity) On the other hand, they were homogeneously disentangled with a tweezers and then their shapes were fixed by thermal compression to adjust the porosity at 97 % (high porosity). The fabric disks prepared were pre-wetted in 70 vol% ethanol aqueous solution overnight to sterilize and enhance their water uptake. To remove ethanol, they were completely rinsed 2 times with PBS and the culture medium for 1 hr.

Isolation and culture of human fat-derived stem cells

Human fat-derived stem cells derived from human fat tissue were primarily isolated from the human fat tissue that was obtained in the reduction mammaplastic surgery of breast cancer patients with informed consent at Kyoto University Hospital. The fat tissue was washed with PBS to carefully remove blood cells, then minced, and digested by 520 U/ml collagenase (Nitta Gelatin Inc., Osaka, Japan) in a water bath at 37 ℃ for 60 min under shaking. The digested tissue was suspended in Medium 199 containing 10 vol% fetal calf serum (FCS), followed by centrifugation ($200 \times g$, 5 min at 4 ℃) to remove the supernatant. After washed twice with the medium, the cells obtained were cultured in a cell-culture flask (75cm^2, Corning Inc., NY 430720, 1×10^3 cells / cm^2) in the medium containing 0.1 μg / ml of bFGF at 37 ℃ and a 5% CO_2 - 95% air atmosphere pressure. The medium was exchanged next day and every 3 days thereafter. When the cells became subconfluent, usually after about 7-day culture, they were detached from the flask by treatment for 5 min at 37 ℃ with the solution of 0.25 % trypsin and 0.02 % EDTA in 100 mM of PBS. Then, the cells were subcultured at a density of 2×10^4 cells/cm^2 and were used at subconfluence for the following experiments.

Cell seeding by static and agitated methods

Two different methods were used to seed the cells into PET non-woven fabrics. Briefly,

500 μl of a cell suspension (2×10^6 cells/ml) was poured onto each non-woven fabric in a polypropylene tube (IWAKI Glass Co. Ltd., Funabashi, Chiba, Japan) (static seeding method). For the agitated seeding method, a non-woven fabric was placed in the cell suspension, followed by agitation with an orbital shaker (Bellco Glass, Inc. Vineland, NJ) at 300 rpm for 6 hr. The cell-seeded non-woven fabrics were thoroughly washed with PBS to exclude non-adherent cells for following cell culture experiments.

Proliferation and osteogenic differentiation by static, agitated, and stirred culture methods

Cell culture was performed by the static, stirred, and agitated methods. For the static and agitated culture methods, cell-seeded non-woven fabrics with the agitated seeding method were placed in each well of a 12-well multiwell culture plate (IWAKI Glass Co. Ltd.) with 3 ml of the culture medium. The cells were cultured under a static condition or agitating on the orbital shaker at 50 rpm. The medium was exchanged every other day. For the stirred culture, six cell-seeded non-woven fabrics with the agitated seeding method were pierced by a 22-gauge needle at the 10 mm distance apart and placed in a spinner flask (1l, Bellco glass Inc., Vineland, NJ). The spinner flask was filled with 700 ml of the culture medium and the half volume was exchanged every 4 days. The similar culture experiment was performed for osteogenic differentiation of cells except that the medium contains 10 nM dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate.

Histological evaluation

The cell-attached non-woven fabrics were fixed in a 10 wt% neutral-buffered folmalin solution, conventionally dehydrated with a series of ethanol aqueous solution at different mixing ratios, immersed in xylene, and embedded in paraffin. The cross-section of samples (5 μm thickness) was prepared at the central portion of disk and stained by Hematoxylin and Eosin while the picture of cross-section was taken by a light microscope (AX-80-64FLBD, Olympus Optical Co., Ltd., Tokyo, Japan) (12 pictures / section). A half area of the cross-section at the outer surrounding portion was defined as the outer portion, while another half was defined as the inner portion. The number of cells present in the inner and outer portions in the fabric was counted and the ratio of cell number in the inner portion to in outer portion was calculated. If the ratio is less than one, it implies that there are more cells in outer portion than in the inner portion. At the ratio close to one, the cells distributed more uniformly in non-woven fabrics.

Measurement of cell number

The number of cells in the non-woven fabric was determined by the fluorometric

quantification of cellular DNA according to the assay reported by Otto et al [17]. Briefly, cell-seeded non-woven fabric specimens were washed with PBS and stored at −30 ℃ until assayed. After thawing, the cells in the specimen were lysed in a buffer solution (pH 7.4) containing 0.2 mg/ml sodium dodecylsulfate (SDS) and 30 mM sodium citrate buffered saline solution (SSC) at 37 ℃ for 12 hr with occasional mixing. The cell lysate (100 μl) was mixed with 400 μl of the SSC buffer. After mixing with 500 μl of a dye solution containing 30 mM SSC and 1 μg/ml Hoechst 33258 dye (12B4809 Nacalai Tesque, Inc., Kyoto, Japan), the fluorescent intensity of mixed solution was measured on a fluorescence spectrometer (F-2000 series, Hitachi Ltd., Tokyo, Japan) at excitation and emission wavelengths of 355 and 460 nm. The number of cells was estimated by a calibration curve between the fluorescent intensity and cell number prepared by use of cells at known cell number. Experiments were done triplicately for each group unless otherwise mentioned.

Measurement of ALP activity

The ALP activity of cells was assayed by a p-nitrophenylphosphate method [18]. Briefly, after the cell samples were lysed by the same procedure described previously, the cell lysate (10 μl) was mixed with 100 μl of 6.7 mM p-nitrophenyl phosphate aqueous solution, followed by leaving at 37 ℃ for 15 min. After mixing with 1 ml of 20 mM NaOH aqueous solution, the absorbance of the solution mixture was measured at 405 nm by a spectrophotometer (DU 650, Beckman Coulter Inc., California). The activity was assessed by the calibration curve prepared by the standard enzyme solution.

Statistical analysis

All the data were analyzed by the Fisher's LSD test for multiple comparison and statistical significance was accepted at $p < 0.05$. Experimental results were expressed as the mean ± the standard deviation of the mean (S.D.).

RESULTS

Cell seeding on PET non-woven fabrics by static and agitated culture methods

Figure 1 shows the number of cells attached to various non-woven PET fabrics with high and low porosities. Irrespective of the PET diameter and porosity, the number of cells attached by the agitated seeding method was large compared with that by the static seeding method. The larger number of cells was attached for the fabrics prepared from the fiber with diameters of 9.0 μm or

larger, while the number of cells attached was the largest at a fiber diameter of 22.0 μm.
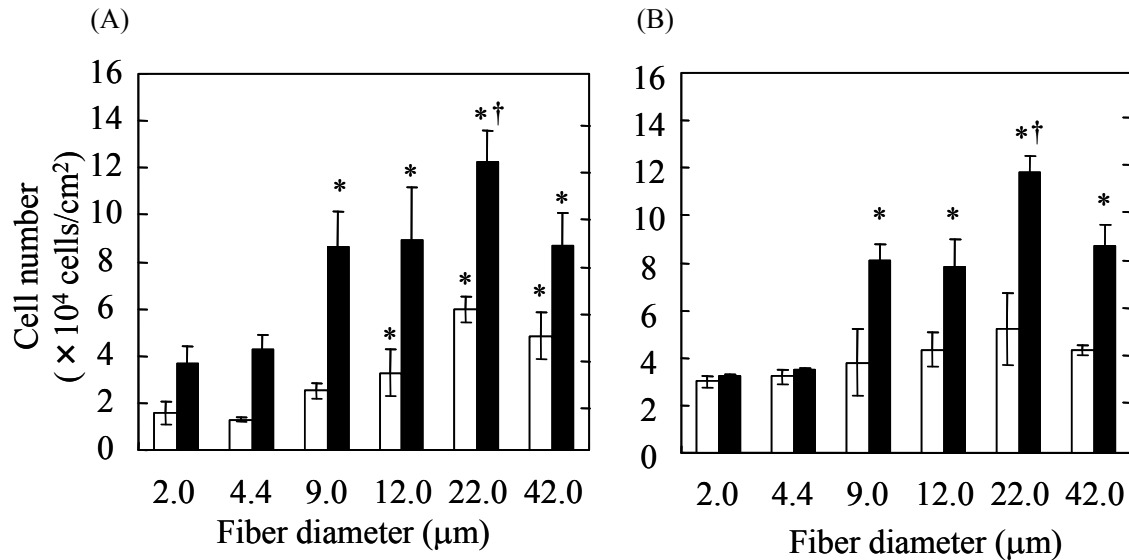
(A)

(B)

Figure 1. The number of cells attached to non-woven fabrics prepared from the PET fiber of various diameters with low (A) or high porosity (B) 6 hr after cell seeding with the static (□) and agitated methods (■).
*, $p < 0.05$; significant against the cells attached to non-woven fabrics with a fiber diameter of 2.0 and 4.4 μm by the corresponding seeding method.
†, $p < 0.05$; significant against the cells attached to non-woven fabrics with a fiber diameter of 9.0, 12.0, and 42.0 μm at the corresponding seeding method.

Figure 2 shows the scanning electron micrographs of cells attached to the non-woven fabrics with various fiber diameters by the static and agitated seeding methods. There was no difference in the shape of cells between the two seeding methods. However, the fiber diameter affects the cell shape. The shape of cells attached on the fibers with 2.0 μm diameter was spherical. The cell morphology became flat as the fiber diameter increased.

Figure 3 shows the distribution of cells attached on the fibers of non-woven fabrics. From the histological section, the number of cells in the outer and inner portions of the section was counted and the inner/outer ratio of cell number was calculated. It seems that the agitated seeding method was effective in achieving homogeneous distribution in the non-woven fabrics compared with the static seeding method. For the fabrics prepared from the fiber with diameters of 2.0 and 4.4 μm, the static method was not effective in cell seeding.
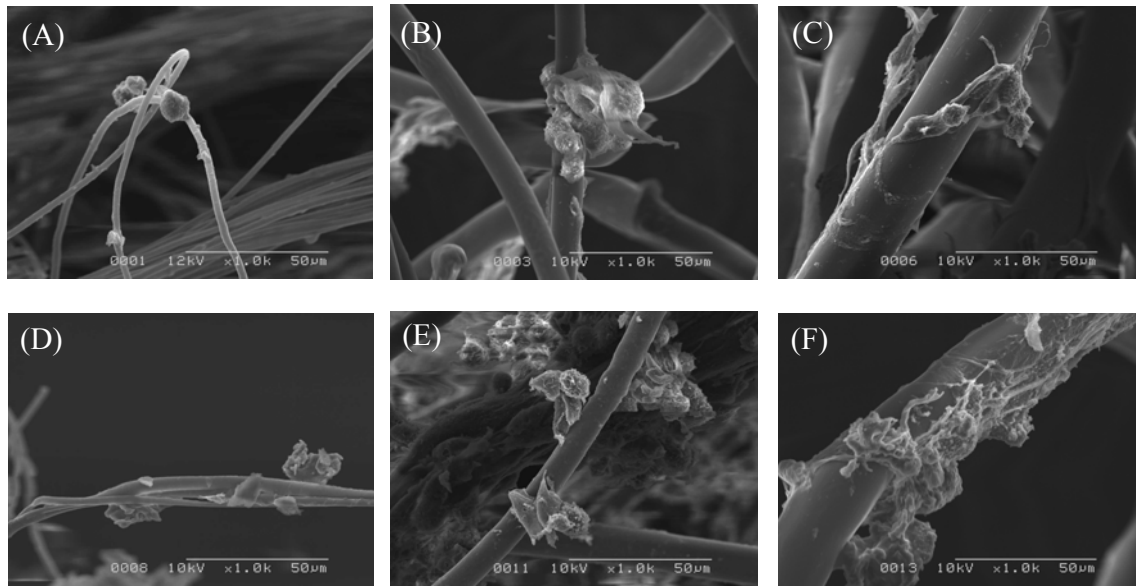
Figure 2. Scanning electron micrographs of cells attached to non-woven fabrics prepared from the PET fiber with diameters of 2.0 (A and D), 9.0 (B and E), and 22.0 μm (C and F) 6 hr after cell seeding with the static (A, B, and C) and agitated methods (D, E, and F).
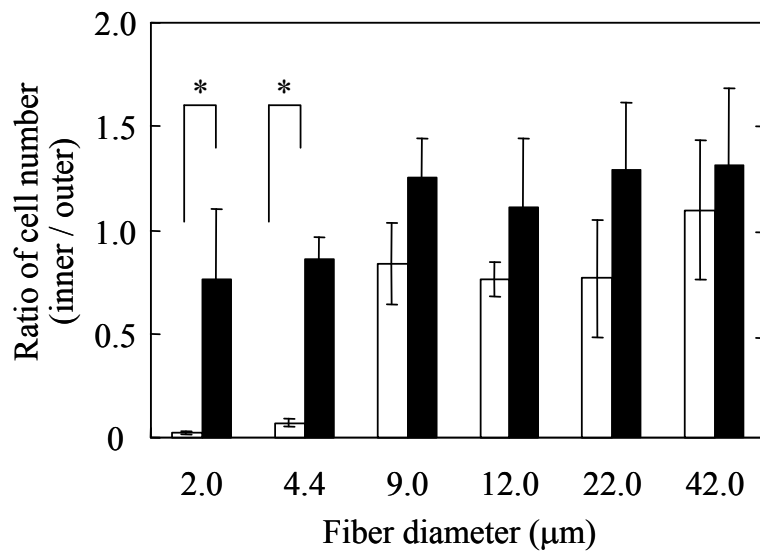

Figure 3. Distribution of cells attached in non-woven fabrics prepared from the PET fiber of various diameters 6 hr after cell seeding with the static (□) and agitated methods (■).
*, $p < 0.05$; significant between two values indicated.

Proliferation of cells cultured on PET non-woven fabrics by static, agitated, and stirred culture methods

Figure 4 shows the proliferation profile of cells in the non-woven fabrics with various fiber diameters and porosities by different culture methods. For the static culture method, the cell number tended to increase for the initial 7 days of culture, but thereafter decreased. The proliferation rate was large when the fiber diameter was 9.0 μm or larger, irrespective of the fabric porosity. On the contrary, by the agitated and stirred culture methods, the larger number of cells was observed than that of the static culture method. The cells cultured by the stirred method continued to proliferate until to 14 days and the cell number did not decrease, which is different from the proliferation profile for other culture methods.
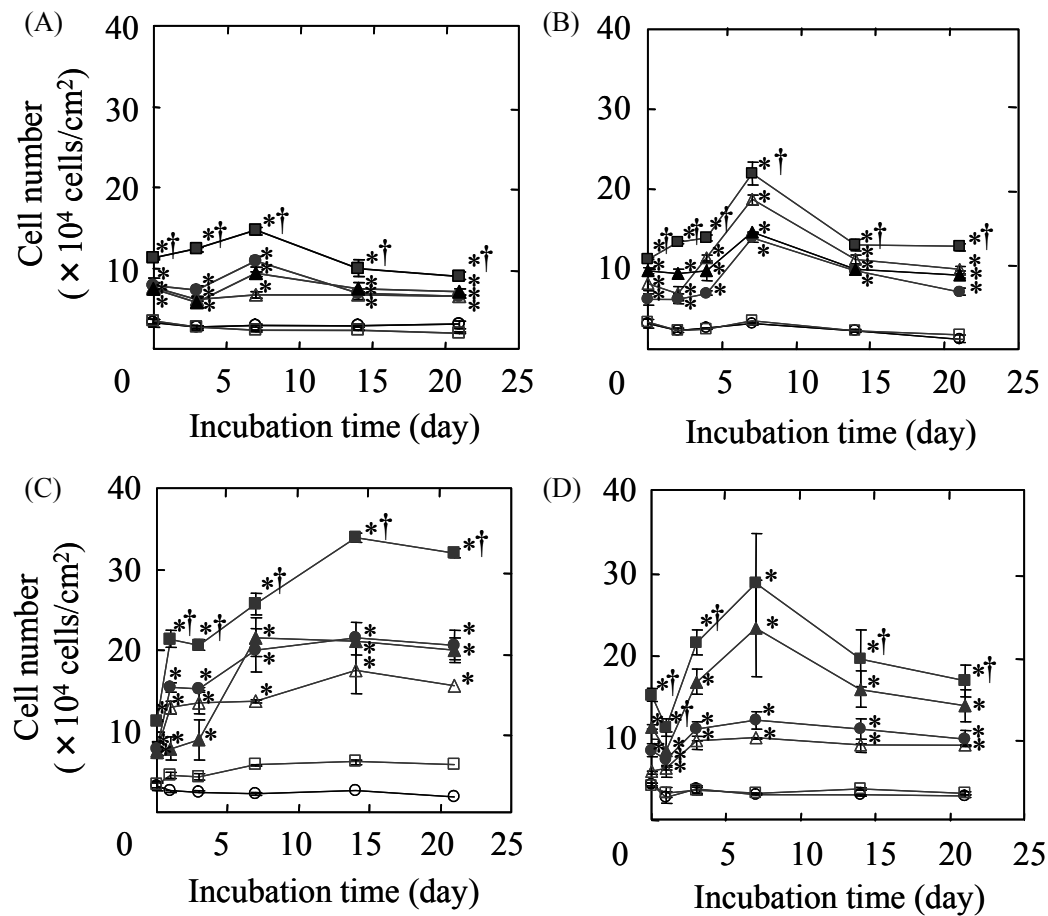

Figure 4. Time course of cells proliferation in non-woven fabrics prepared from the PET fiber with various diameters of 2.0 (○), 4.4 (□), 9.0 (△), 12.0 (●), 22.0 (■), and 44.0 μm (▲) by the static (A and B), stirred (C), and agitated culture methods (D). The fabric porosity is low (A, C, and D) or high (D).
*, p<0.05; significant against cells adhered to non-woven fabrics with fiber diameter of 2.0 and 4.4 μm on the corresponding day.
†, p<0.05; significant against adhered to non-woven fabrics with fiber diameter of 9.0, 12.0 and 42.0 μm on the corresponding day.

Figures 5 and 6 show the histological sections of cells attached to non-woven fabrics with a fiber diameter of 22.0 μm and the kinetics of cell distribution in the non-woven fabrics. The number of cells in the inner portion of non-woven fabrics decreased with time for the static and agitated culture methods although the extent was greater for the former than the latter. However, for the stirred culture method, the cells proliferated uniformly and the number of cells increased with time.
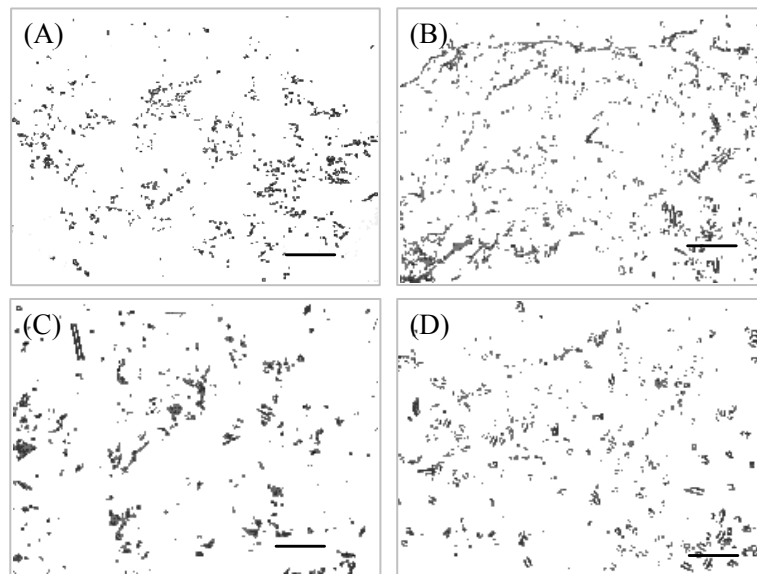

Figure 5. Distribution of cells attached and proliferated in non-woven fabrics prepared from the PET fiber with a diameter of 22.0 μm 6 hr after seeding (A) or further cultivation for during 21 days by the static (B), stirred (C), and agitated methods (D). The bar is 500 μm.
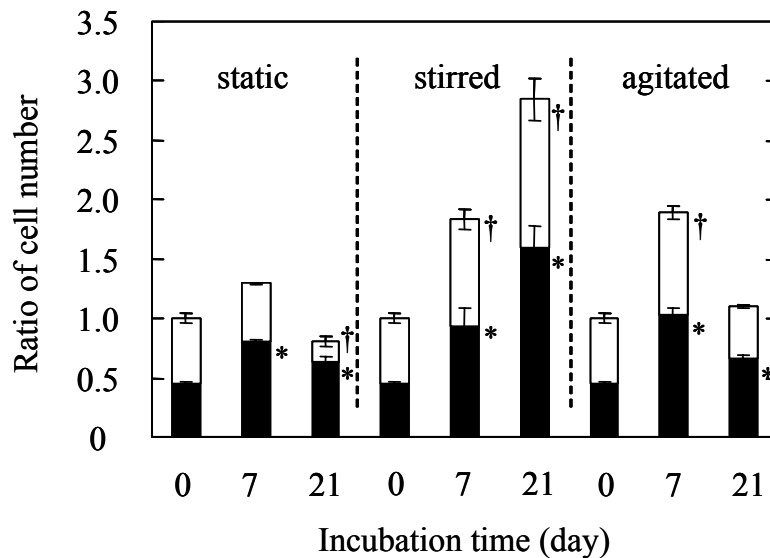

Figure 6. Time course of cell distribution in the outer (□) or inner portion (■) of non-woven fabrics prepared from the PET fiber with a diameter of 22.0 μm. The total number of cells proliferated is normalized by that of cells initial attached.
*, $p < 0.05$; significant against the number of cells initially attached in the outer portion.
†, $p < 0.05$; significant against the number of cells initially attached inner portion.

Osteogenic differentiation of cells cultured on PET non-woven fabrics by static, agitated, and stirred culture methods

Figure 7 shows the ALP activity of cells on the non-woven fabrics with various fiber diameter and porosities after osteogenic differentiation culture for 3 weeks. The ALP activity was large for cells cultured in the differentiation medium compared with those in the normal medium, irrespective of the fiber diameter. No effect of the fiber diameter on the ALP activity was observed, irrespective of the fabric porosity. The ALP activity is the lowest of cells cultured by the stirred culture method while the highest was observed for cells cultured by the static culture method.
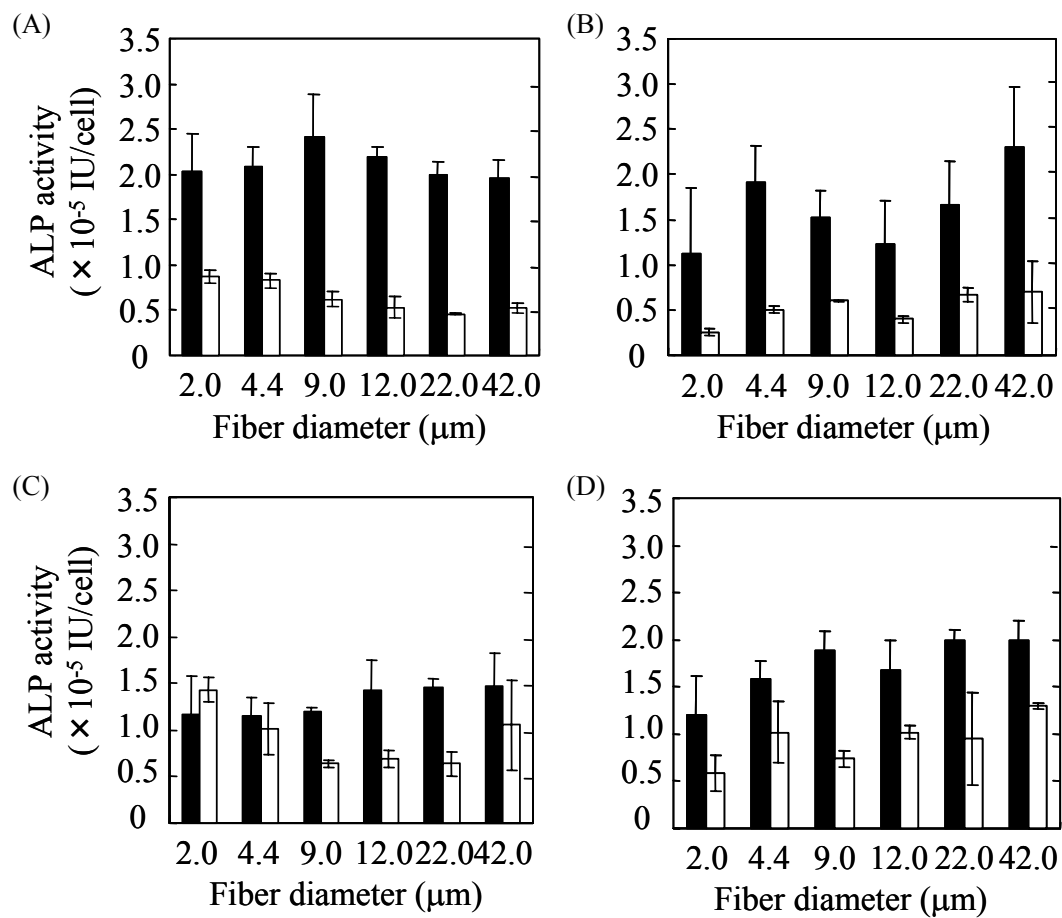

Figure 7. ALP activity of cells cultured in non-woven fabrics prepared from the PET fiber with various diameters with low (A) or high porosity (B) for 3 weeks in the normal (□) and bone differentiation medium (■) by the static (A and B), stirred (C), and agitated method (D).

DISCUSSION

The present study demonstrates that the fiber diameter and culture method affect the proliferation and osteogenic differentiation of human fat-derived stem cells in the non-woven fabrics. The fiber diameter affected the initial adhesion and proliferation of cells although it did not affect the osteogenic differentiation. The cell proliferation and differentiation were also influenced by the culture method.

The agitated seeding method was effective in enhancing the number of cells attached and homogeneity of cell distribution in the fabrics. This can be explained in terms of high frequency of cells adhesion to the fabric fibers. It is possible that the agitation of cell suspension enabled cells to penetrate into the fabrics as well as enhance the frequency of their collision to the fabrics, resulting in enhanced cell number and the distribution homogeneity.

The number of cells initially attached was the largest for the non-woven fabrics with a fiber diameter of 22.0 μm. This can be attributed to the large surface area of cells to attach. As shown in Figure 2, the cells attached in a spherical shape on the PET fiber with 2.0 μm diameter. It is conceivable that the surface area of fibers was too small to allow the cells to completely attach to the fiber surface. Such an unstable adhesion would result in decreased number of cells initially attached. Poor proliferation of cells on the fiber with diameters 4.4 μm or smaller can also be explained by the unstable cell adhesion. From the viewpoint of cell proliferation, the larger the fiber diameter, the better the cell proliferation is. This is because that for the fibers with larger diameter, cells would be able to utilize the large surface of fibers for effective proliferation. However, the number of cells initially attached to the fabrics with a fiber diameter of 42.0 μm was smaller than that of fabrics with 22.0 μm fiber. It has been well recognized that cells attached in different fashions onto the substrate surface with different shapes and sizes [19-21]. It is possible that cells recognize the curvature of fiber when attached. The reason is not clear at present. However the ALP activity, which is a bone differentiation marker, did not depend on the fiber diameter, irrespective of the culture method and the fabric porosity. Once attached and proliferated, the cells may osteogenically differentiated, irrespective of the substrate shape. The ALP activity increased with an increase in the number of cells proliferated while the ALP activity per cells was similar, irrespective of the type of culture substrates (unpublished data).

Proliferation and osteogenic differentiation were influenced by the culture methods. For the static culture method, the cell proliferation was not good, but the differentiation was good. For the stirred culture method, the cell proliferation was good, but the differentiation was not good. For the agitated culture method, both the cell proliferation and differentiation were in the middle level of the two methods. The difference in the proliferation behavior may depend on the

circulation of culture medium throughout the fabrics. In the static culture method, since the medium circulation in the fabrics is not expected, it is likely that the cell proliferation was not good. On the contrary, the stirred culture method would bring about better medium circulation in the non-woven fabrics. As the result, the nutrients and oxygen would effectively supply to cells while the wastes would be washed out of the fabrics. Some researches have demonstrated that the higher extent of differentiation is due to the suppressed cell proliferation [22]. The present correlation between the cell proliferation and differentiation were similar to that reported previously.

For the static and agitated culture methods, cells proliferated until 7 days after seeding, but thereafter the cell number reduced. This can be explained in terms of medium circulation. It is likely that the medium circulation was so poor that cells weakened or died for a long-term culture. The number of cells proliferated in the fabrics with low porosity was smaller than that of fabrics with high porosity. It is possible that the low porosity made worse the medium circulation in the fabrics, resulting in suppressed cell proliferation.

Several bioreactor systems have been explored to achieve the uniform sterically homogeneous distribution and proliferation of cells inside the scaffold [23-29]. In the present study, the static culture, as expected, allowed the cells to proliferate only at the outer portion of scaffold. It is likely that the cells proliferated only around the fabric surface because of enough oxygen and nutrition since the in-flux and out-flux of culture medium for the fabrics was not sufficient for cell proliferation. Sikavitsas et al. have demonstrated that a spinner flask culture (stirred culture) enhanced the proliferation of MSC as well as their ALP activity, osteocalsin, and calcium deposition in a poly (DL-lactic-co-glycolic acid) (PLGA) scaffold [23]. Similarly, this study also indicated that the stirred culture enhanced the proliferation of cells both in the outer and inner portion of fabric scaffold. However, the osteogenic differentiation of cells was not enhanced. This difference may be due to the different type of cells. The agitated culture was also not effective method to enhance the proliferation of cells in the inner portion of fabrics, probably because the medium circulation is not enough as good as that of stirred method. Goldstein et al. have reported that a flow perfusion enhanced the early differentiation and three-dimensional distribution of marrow stromal osteoblasts seeded in the PLGA scaffolds when compared with spinner flask or rotating vessel bioreactor [26]. The rotating vessel or flow perfusion bioreactor will be used to compare the proliferation and differentiation of human fat-derived stem cells between bioreactor systems.

REFERENCES

1. B. Alberts, D. Bray and J. Lewis *Molecular Biology of the Cell* 971 (1994)

2. W. W. Carley, A. J. Milici and J. A. Madri *Exp Cell Res* **178**, 426 (1988)

3. A. J. Putnam and D. J. Mooney *Nat Med* **2**, 824 (1996)

4. C. A. Heath *Trends Biotechnol* **18**, 17 (2000)

5. J. A. Hubbell *Biotechnology (N Y)* **13**, 565 (1995)

6. J. P. Vacanti, R. Langer, J. Upton and J. J. Marler *Adv Drug Deliv Rev* **33**, 165 (1998)

7. H. Shin, S. Jo and A. G. Mikos *Biomaterials* **24**, 4353 (2003)

8. Y. Takahashi and Y. Tabata *Tissue Eng* **9**, 931 (2003)

9. J. N. Beresford, J. H. Bennett, C. Devlin, P. S. Leboy and M. E. Owen *J Cell Sci* **102 (Pt 2)**, 341 (1992)

10. M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig and D. R. Marshak *Science* **284**, 143 (1999)

11. A. I. Caplan *J Orthop Res* **9**, 641 (1991)

12. S. Wakitani, T. Saito and A. I. Caplan *Muscle Nerve* **18**, 1417 (1995)

13. G. Ferrari, G. Cusella-De Angelis, M. Coletta, E. Paolucci, A. Stornaiuolo, G. Cossu and F. Mavilio *Science* **279**, 1528 (1998)

14. P. A. Zuk, M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz and M. H. Hedrick *Tissue Eng* **7**, 211 (2001)

15. P. A. Zuk, M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim and M. H. Hedrick *Mol Biol Cell* **13**, 4279 (2002)

16. S. Gronthos, D. M. Franklin, H. A. Leddy, P. G. Robey, R. W. Storms and J. M. Gimble *J Cell Physiol* **189**, 54 (2001)

17. J. Rao and W. R. Otto *Anal Biochem* **207**, 186 (1992)

18. D. Kobayashi, H. Takita, M. Mizuno, Y. Totsuka and Y. Kuboki *J Biochem* **119**, 475 (1996)

19. A. S. Curtis and P. Clark *Crit Rev Biocompat* **119**, 475 (1990)

20. R. Shnghvi, G. N. Stephanopoulos and D. I. Wang *Biotechnol Bioeng* **43**, 764 (1994)

21. A. F. von Recum and T. G. van Kooten *J Biomater Sci Polym Ed* **7**, 181 (1995)

22. S. P. Baldwin and W. M. Saltzman *Tissue Eng* **7**, 179 (2001)

23. V. I. Sikavitsas, G. N. Bancroft and A. G. Mikos *J Biomed Mater Res* **62**, 136 (2002)

24. L. E. Freed and G. Vunjak-Novakovic *Principles of Tissue Engineering* 143 (2000)

25. E. A. Botchwey, S. R. Pollack, E. M. Levine and C. T. Laurencin *J Biomed Mater Res* **55**, 242 (2001)

26. A. S. Goldstein, T. M. Juarez, C. D. Helmke, M. C. Gustin and A. G. Mikos *Biomaterials* **22**, 1279 (2001)

27. G. N. Bancroft, V. I. Sikavitsas, J. van den Dolder, T. L. Sheffield, C. G. Ambrose, J. A. Jansen and A. G. Mikos *Proc Natl Acad Sci U S A* **99**, 12600 (2002)

28. C. M. Begley and S. J. Kleis *Biotechnol Bioeng* **70**, 32 (2000)

29. J. Glowacki, S. Mizuno and J. S. Greenberger *Cell Transplant* **7**, 319 (1998)

Chapter 6

Adhesion and proliferation of human fat-derived stem cells on two- and three-dimensional PET substrates modified with RGD peptide

INTRODUCTION

Considering in vivo conditions, cells adhere onto the three-dimensional (3D) substrates of ECM. Based on this, it is no doubt that the 3D culture gives cells better conditions for their proliferation and differentiation than the culture on the two-dimensional (2D) substrate. Various types of 3D substrates have been used for the experiments of cell culture [1-4]. It is known that the cell culture is influenced by the bulk properties of 3D substrates, including the porosity, pore size, and thickness of sponges and non-woven fabrics or the fiber diameter of fabrics [2-4]. These bulk properties affect the adhesion, proliferation, and differentiation of cells. In addition, cells behavior for the substrate depends on the cell cultivation technique, such as cell seeding and culture methods. An agitated seeding method enabled cells to seed homogeneously inside of substrates and consequently enhance the number of cells proliferated compared with a static method [5]. Culture methods gave cells better conditions for the proliferation. An agitated culture enhanced the proliferation of fat-derived stemcells in the PET non-woven fabrics to a significantly great extent compared with a static methods [6].

Various comparison studies between the 2D and 3D substrates have been investigated in terms of adhesion, proliferation, and differentiation of cells [7-9]. However, since the researches have been done to examine the cell behavior for different volumes of culture medium, the seeding densities of cells, and the culture methods, it is practically impossible to directly compare it between the 2D and 3D substrates.

For 2D substrates, the surface property has been modified chemically, physically, and biologically by various methods, such as the graft polymerization, oxidation, coating, and roughening or the immobilization of substances to induce the adhesion, proliferation, and differentiation of cells [10]. Treatment of PET with NaOH solution increases the number of carboxyl and hydroxyl groups on the surface by the chemical breakage of PET main chain [11]. A tripeptide motif, RGD, is sequenced in the ECM molecule, such as fibronection, vitronectin, collagen, and laminin, and interacted with the integrin receptor of cells. RGD is immobilized to induce the cell adhesion on substrates [12]. The RGD modification has been used for 3D substrates to induce the adhesion, proliferation, and differentiation of cells [13-15].

This Chapter investigates the adhesion and proliferation of human fat-derived stemcells

for the PET films of 2D substrate and non-woven fabrics of 3D substrate. The surface of PET non-woven fabrics was treated by NaOH to allow to generate the carboxyl groups, and then RGD was chemically immobilized through the groups onto the fabrics surface. Human fat-derived stemcells were seeded at the same cell density and cultured at the same ratio of medium volume to the surface area on the PET films and non-woven fabrics to compare the cell adhesion and proliferation between the two substrates. We examine the effect of the cell seeding and culture method on the cell behavior for the 2D and 3D substrates.

EXPERIMENTAL

Preparation of PET substrates treated by NaOH

PET films and non-woven fabrics with a fiber diameter of 24 μm were kindly supplied by Teijin Ltd. (Tokyo, Japan) and Toray Inc. (Shiga, Japan), respectively. The PET films and non-woven fabrics were washed with methanol in an ultrasound bath (Brason 2510, Yamato Tokyo, Japan), followed by rising with double-distilled water (DDW). The PET non-woven fabric was compressed between two glass plates at 150 $^{\circ}$C for 30 min to adjust the porosity at 94% and the thickness of 1.5 mm. The film was punched out into 14-mm-dimater disks, while the PET non-woven fabric was cut into 6-mm-diamter disks.

The PET film and non-woven fabric disks (1 g) were hydrolyzed with 1N NaOH solution (100 ml) at 60 $^{\circ}$C for 1 hr to generate the carboxyl and hydroxyl groups by the chemical breakage of PET main chain [11]. The NaOH-treated PET disks were washed with DDW.

Characterization of NaOH-treated PET substrates

The PET film disks were characterized by measuring the water contact angle and the density of carboxyl groups. The water contact angle was measured with a geometer (Contact angle meter CA-X, Kyowa Interface Science, Saitama, Japan) according to the method previously reported [16]. Briefly, a water drop (9 μl) was dropped onto the surface of PET films at room temperature and at the same timing after water dropping, the water contact angle was measured independently 15 times for each sample.

The density of carboxyl groups was measured according to the conventional method of Toluidine Bule O (TBO) adsorption [17]. The PET disks were immersed in 2.5×10^{-4} M of TBO aqueous solution at pH 10 for 2 hr. After washing with NaOH aqueous solution (pH 10) to remove free TBO molecules, the disks treated with TBO were placed in 50 v/v% acetic acid solution to extract the TBO adsorbed from the disks. The absorbance of the solution was measured by a

microplate reader at a wavelength of 633 nm (Versa max, Molecular Device Japan Co., Osaka, Japan) and the carboxyl groups were determined by the calibration curve of concentration-absorbance of TBO.

Immobilization of RGD on NaOH-treated PET substrates

RGD peptide (GGGRGDSP), kindly supplied by Faculty of Engineering, Osaka Institute of Technology, was immobilized on the NaOH-treated PET non-woven fabrics [18, 19]. Briefly, the disks were sterilized by 70 vol% ethanol aqueous solution, and then washed with phosphate-buffered saline solution (100 mM, pH 7.4, PBS). Next, they were immersed in sterilized PBS solution (250 μl/surface area cm^2) containing 0.2 M of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.05 M of N-hydroxysuccinimide (NHS) for 20 min. After washing five times with PBS, the disks were treated with sterilized PBS solution (250 μl/surface area cm^2) containing 20 μg/ml of RGD peptide. After RGD immobilization for 2 hr at 37°C, the PET disks were washed with PBS five times to exclude non-coupled RGD peptide from the RGD immobilized disks.

Measurement of density of RGD immobilized

The amount of RGD peptide immobilized was measured by a radiolabeling method. RGD peptide (GGGRGDSY) was radioiodinated according to the conventional chloramine-T method [20]. Briefly, 10 μl of Na^{125}I (PerkinElmer Japan Co. Ltd., Kanagawa, Japan) was added to 400 μl of 1 mg/ml RGD solution in 0.5 M potassium phosphate-buffered solution (pH 7.5) containing 0.5 M sodium chloride (reaction buffer). Then, 200 μl of the reaction buffer containing 0.2 mg/ml of chloramine-T was added to the solution mixture. After agitating at room temperature for 2 min, 200 μl of PBS containing 4mg/ml of sodium metabisufate was added to the solution mixture to stop the raioiodination. The reaction mixture was passed through a column (Dowex I-X8, Muromachi Technology Co. Ltd., Tokyo, Japan) to remove the uncoupled ^{125}I molecule from the ^{125}I-labeled RGD. ^{125}I-labeled RGD was used to immobilize for the PET non-woven fabrics with or without NaOH treatment. The radioactivity of RGD immobilized for the PET disks was measured by a γ counter (Auto Well Gamma System; Aloka, Tokyo, Japan). In addition, the concentration of ^{125}I-labeled RGD peptide was determined by Micro BCA protein assay kit (#HJ106352, Pierce, Thermo Fisher Scientific Inc., IL). From both the concentration and radioactivity, the amount of RGD immobilized was determined. The similar reaction produce was done for RGD without radioiodination and the RGD immobilized PET disks were used for the following cell culture experiment.

Isolation and culture of human fat-derived stem cells

Fat-derived stemcells were primarily isolated from the human adipose tissue that had been collected in the reduction mammaplastic surgery of breast cancer patients receiving the informed consent at Kyoto University Hospital. The adipose tissue (5 ml) after removing cancer and fibrous tissues was washed with PBS to carefully remove blood cells, then minced, and digested by 520 U/ml collagenase (Nitta Gelatin Inc., Osaka, Japan) at 37 $^{\circ}$C for 15 min under shaking [21]. The digested was suspended in Medium 199 containing 10 vol% FCS and 1 vol% penicillin-streptomycin (normal medium). The suspension was filtered through a 200-μm nylon mesh and centrifuged at 1,000 rpm for 5 min at 4 $^{\circ}$C. After washing twice with the normal medium, the cells obtained were suspended in the medium and cultured at 37 $^{\circ}$C in a 75-cm^2 culture flask (#430720, Corning Inc., New York). Non-adherent cells were removed 1 day later and adherent cells were proliferated in the nomal medium containing 1 μg/ml of basic fibroblast growth factor (bFGF) kindly supplied from Kaken Pharmaceutical Co., Ltd., Tokyo, Japan [21]. After incubation for 7 to 10 days, the cells density became almost confluent. The cells proliferated were detached with 0.25 wt% trypsin solution containing 7 μM ethylenediaminetetraacetate in PBS and used for the following experiments.

Cell seeding and proliferation on two- and three-dimensional PET substrates by static and agitated culture methods

Fat-derived stemcells were seeded onto the PET film disks by a static seeding method and onto the PET non-woven fabric disks by the static or agitated seeding method at the density of 1×10^4 cells/cm^2. Briefly, 500 μl of cell suspension solution (4×10^4 cells/ml) was placed onto the PET film disks in each well of 24-well multiwell dishes (#1520703, 2 cm^2, non-treated multiwell plate, Asahi Techno Glass Co., Tokyo, Japan) or the cell culture dish (#23007001, Corning Inc.) as a control [21]. For the static seeding, 75 μl of cell suspension (1.3×10^6 cells/ml) was placed onto each PET non-woven fabric disk which had been placed in each well of 96-well multiwell plates (#22207007, 0.3 cm^2, Corning Inc.). For the agitated seeding, 500 μl of cell suspension (1.9×10^5 cells/ml) was poured onto PET non-woven fabric disks with or without NaOH treatment or RGD immobilization in a polypropylene tube (Greiner Bio-one Co., Ltd, Tokyo, Japan), followed by agitation with an orbital shaker (Bellco Glass, Inc. Vineland, NJ USA) at 300 rpm for 6 hr [6].

After 6 hr incubation following the cell seeding, cells were cultured at the same ratio of medium volume to the surface area on the PET films (≈ 2 cm^2) and non-woven fabrics (≈ 9.5 cm^2). As a control, cells seeded by the static seeding method on PET film disks or the cell culture dish were cultured by the static culture method in 500 μl of the normal medium. For cells seeded on PET non-woven fabric disks by static seeding method, the cells were cultured in 2,375 μl of the normal medium by the static culture method after plating into each well of 24-well multiwell plate

(#1520703, 2 cm^2, non-treated multiwell plate, Asahi Techno Glass Co.). For cells seeded on PET non-woven fabric disks with or without NaOH treatment or RGD immobilization by agitated seeding method, the cells were cultured in 2,375 µl of the normal medium by the agitation culture method with an orbital shaker (Bellco Glass, Inc.) at 50 rpm [6].

Observation of alive cells

The cells cultured on various PET disks were stained by the live/dead viability cytotoxicity assay kit (#42830A, Molecular Probes, Invitrogen Co., CA) to determine alive and dead cells [22]. The cells incubated with PET disks for 6 hr or 15 days were washed with PBS five times and incubated with the solution in kit containing fluorescence-labeled calcein (Ex:495 nm, Em:515 nm) and ethidium homodimer (Ex:495 nm, Em:635 nm) for 5 min. After washing with PBS five times, the stained cells were observed by confocal laser scanning microscope (Olympus Fluroview FV300 confocal laser scanning microscope, Olympus Optical Co.).

Measurement of cell number

The number of cells proliferated was measured by a DNA assay method [23]. The cells were rinsed twice with PBS, underwent the conventional freeze-thaw process, and finally incubated in 1 ml of aqueous solution containing 0.2 mg/ml sodium dodecyl sulfate, 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate for 1 hr to completely lyze cells. The cell lysate (100 µl) was mixed with 100 µl of 1 µl/ml Hoechst 33258 dye solution (bisbenzimide H33258 fluorochrome trihydrochloride dimethyl sulfoxide solution, #L2E4809, Nacalai Tesque, Inc., Kyoto, Japan) containing 9.0 mg/ml NaCl and 4.4 mg/ml sodium citrate. The fluorescence intensity of mixed solution was measured on a fluorescence spectrophotometer (Spectra Max Gemini Em, Molecular Device Japan Co., Osaka, Japan) at exciting and emission wavelengths of 355 and 460 nm. The cell number was determined by the calibration curve prepared from the fluorescent measurement for the known number of cells.

Immunobloting of FAK phosphorylation

The cell lysates prepared were mixed with the Radio-Immunoprecipitation Assay (RIPA) buffer containing protease inhibitors (Sigma-Aldrich Co., MO) [24]. The protein content of cell lysates was measured using the BioRad Protein Assay Kit (BioRad Laboratories Inc., CA). The cell lysates were applied to sodium dodecylsulfate polyacrylamide electrophoresis (SDS-PAGE), and transferred to an Immobilon-P membrane (Millipore Co., MA). The membrane was immunoblotted with antibodies against phospho-FAK (upstate, Millipore Co.) and β-actin (Sigma-Aldrich Co.) as an internal control as the diluted ratios of 1/1,000 and 1/10,000, respectively. After incubation in peroxidase-conjugated anti-mouse or anti-rabbit secondary

antibody (Sigma-Aldrich Co.), the target proteins were immunologically visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Thermo Fisher Scientific Inc., IL).

Data analysis

All the data were statistically analyzed by post-hoc Fisher's PLSD tests and expressed as the mean ± the standard deviation of the mean. Statistical significance was accepted at $p < 0.05$

RESULTS

Characterization of NaOH-treated PET substrates

Figure 1 (A) shows the water contact angle of PET film surfaces before and after NaOH treatment. The water contact angle of NaOH-treated PET films was lower than that of the original, non-treated PET films. Figure 1 (B) shows the density of carboxyl groups for the PET films and non-woven fabrics before and after NaOH treatment. The NaOH treatment increased the carboxyl groups. The density of carboxyl groups of NaOH-treated PET films was as same as that of NaOH-treated PET non-woven fabrics.
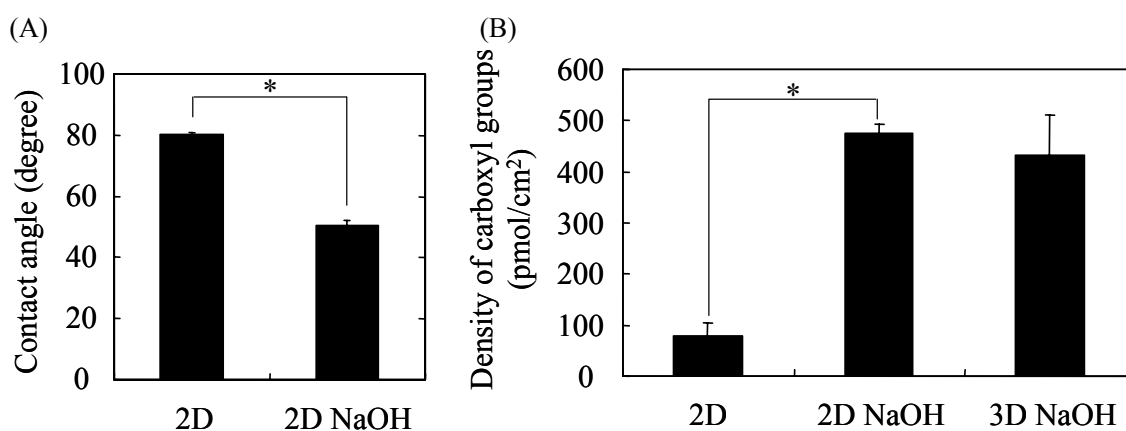
Figure 1. (A) Water contact angles of PET films before (2D) and after NaOH treatment (2D NaOH).
*, p<0.05; significant between the two groups indicated.
(B) Density of carboxyl groups on PET films before (2D) and after NaOH treatment (2D NaOH) or and non-woven fabrics after NaOH treatment (3D NaOH).
*, p<0.05; significant between the two groups indicated.

Density of RGD immobilized on NaOH-treated PET substrates

Figure 2 shows the density of RGD immobilized for PET non-woven fabrics. The density of RGD for the PET non-woven fabrics pretreated with EDC and NHS was higher than that without pretreatment. Little RGD immobilization was observed in the case of neither EDC nor NHS, irrespective of the NaOH treatment.
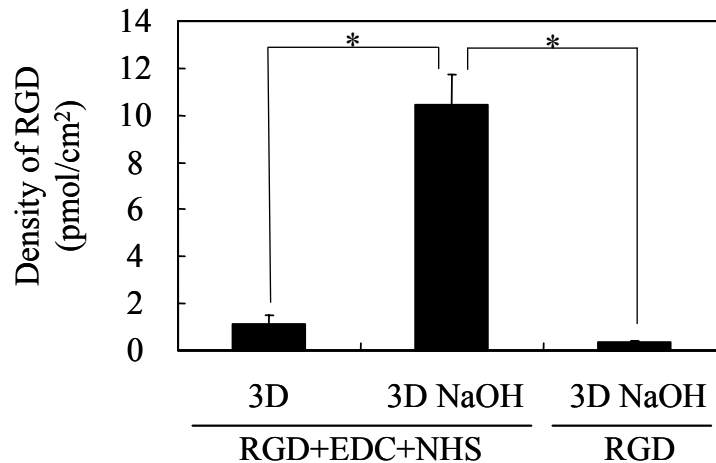

Figure 2. Density of RGD immobilized onto PET non-woven fabrics before (3D) and after NaOH treatment (3D NaOH). RGD was immobilized with or without pretreatment of EDC and NHS.
*, $p < 0.05$; significant between the two groups indicated.

Adhesion of cells cultured on two- or three-dimensional RGD-immobilized PET substrates by static and agitated culture methods

Figure 3 shows the confocal laser microscopic images of fat-derived stemcells following the alive/dead assay. No dead cell was observed for all the PET films and non-woven fabrics. The morphology of cells adhered by the agitated seeding method for PET non-woven fabrics tended to be of round shape, compared with that of cells adhered by the static seeding method. The NaOH treatment and the subsequent RGD immobilization affected the cell morphology and the shape of cells adhered tended to spread, which is similar to that of cells adhered by the static seeding method.

Figure 4 shows the number ratio of fat-derived stemcells 6 hr after seeding. For the static seeding, the number ratio of cells adhered on the PET films was similar to that of the cell culture dish. The cell number ratio was significantly lower for the PET non-woven fabrics than that of the PET films. Comparing for PET non-woven fabrics, the cell number ratio was significantly lower by the static seeding method than that of the agitated seeding method. The NaOH treatment and RGD immobilization increased the cell number ratio.
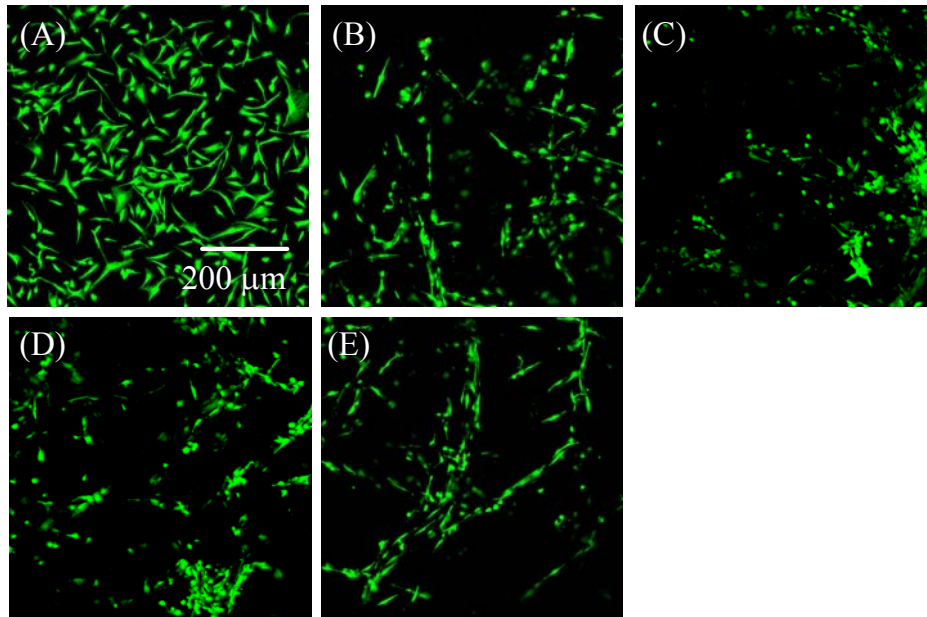
Figure 3. Confocal laser microscopic images of alive/dead assay for fat-derived stemcells on PET films and non-woven fabrics 6 hr after the static (A and B) or agitated seeding method (C, D, and E). The cells were seeded on the PET film (A) and the PET non-woven fabrics (B and C), that NaOH treated (D) or that NaOH treated and RGD immobilized (E).
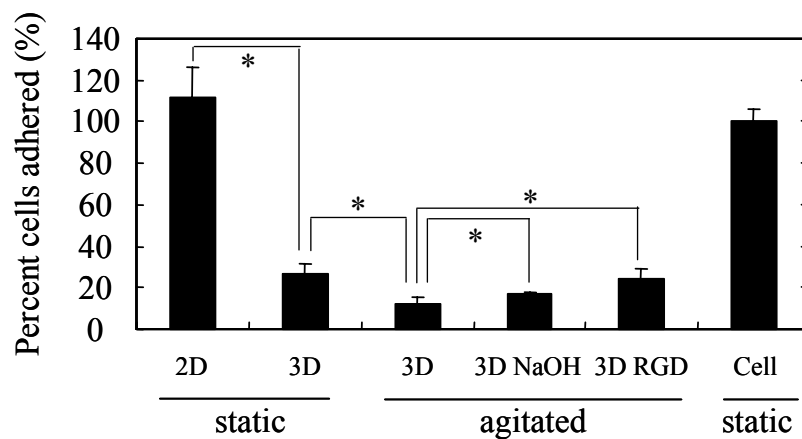

Figure 4. The number of fat-derived stemcells adhered 6 hr after the static or agitated seeding method. The cells were seeded on the PET films (2D) or the PET non-woven fabrics before (3D) and after NaOH treatment (3D NaOH). RGD was immobilized onto the fabrics NaOH treated following the pretreatment of EDC and NHS (3D RGD). 100% indicates the number of cells adhered on a cell culture dish (Cell).
*, $p < 0.05$; significant between the two groups indicated.

Proliferation of cells cultured on two- or three-dimensional RGD-immobilized PET substrates by static and agitated culture methods

Figure 5 shows the confocal laser microscopic images of fat-derived stemcells cultured for 15 days. For the PET non-woven fabrics, cells were observed along the fibers while some dead cells were observed, irrespective of the NaOH treatment and RGD immobilization.

Figure 6 shows the time profile of fat-derived stemcells proliferation. The number of cells cultured on the PET films increased with an increase in the culture time period, similarly to the cell culture dish. For the fabrics, the number of cells increased with time by the static culture method. However, the number was significantly lower than that of PET films. When compared at the number ratio of cells, the value was significantly larger for the PET non-woven fabrics cultured by the agitated culture method than that of PET films and non-woven fabrics by the static culture method at 5, 10, and 15 days. NaOH treatment and RGD immobilization hardly affected the time profile of cell proliferation. However, compared at the number ratio, the value was significantly larger for the PET non-woven fabrics cultured by the agitated culture method than that of NaOH-treated and RGD-immobilized PET non-woven fabrics by the static culture method.
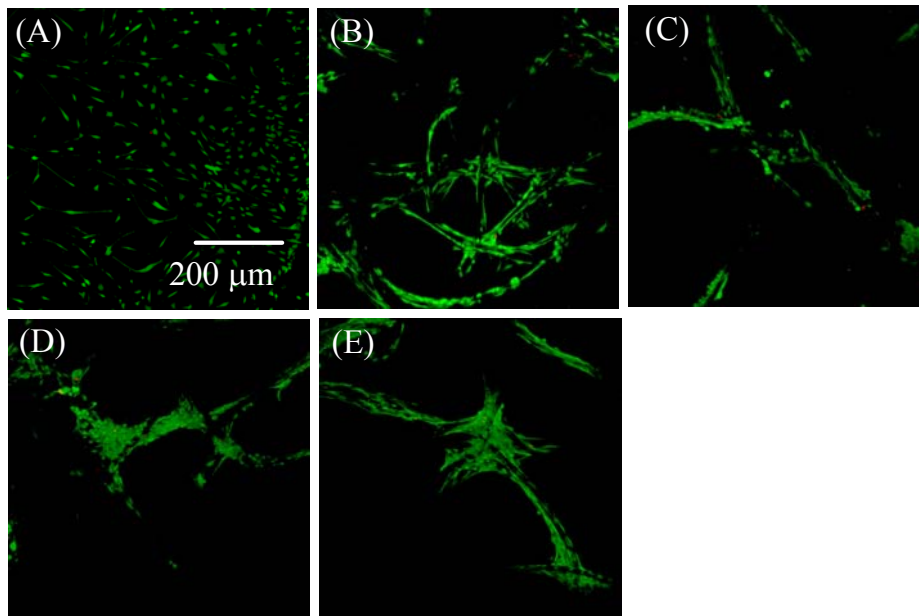

Figure 5. Confocal laser microscopic images of alive/dead assay for fat-derived stemcells proliferated on PET films and non-woven fabrics 15 days after the static (A and B) or agitated seeding and culture (C, D, and E). The cells were seeded and cultured on the PET film (A) and the PET non-woven fabrics (B and C), that NaOH treated (D) or that NaOH treated and RGD immobilized (E).
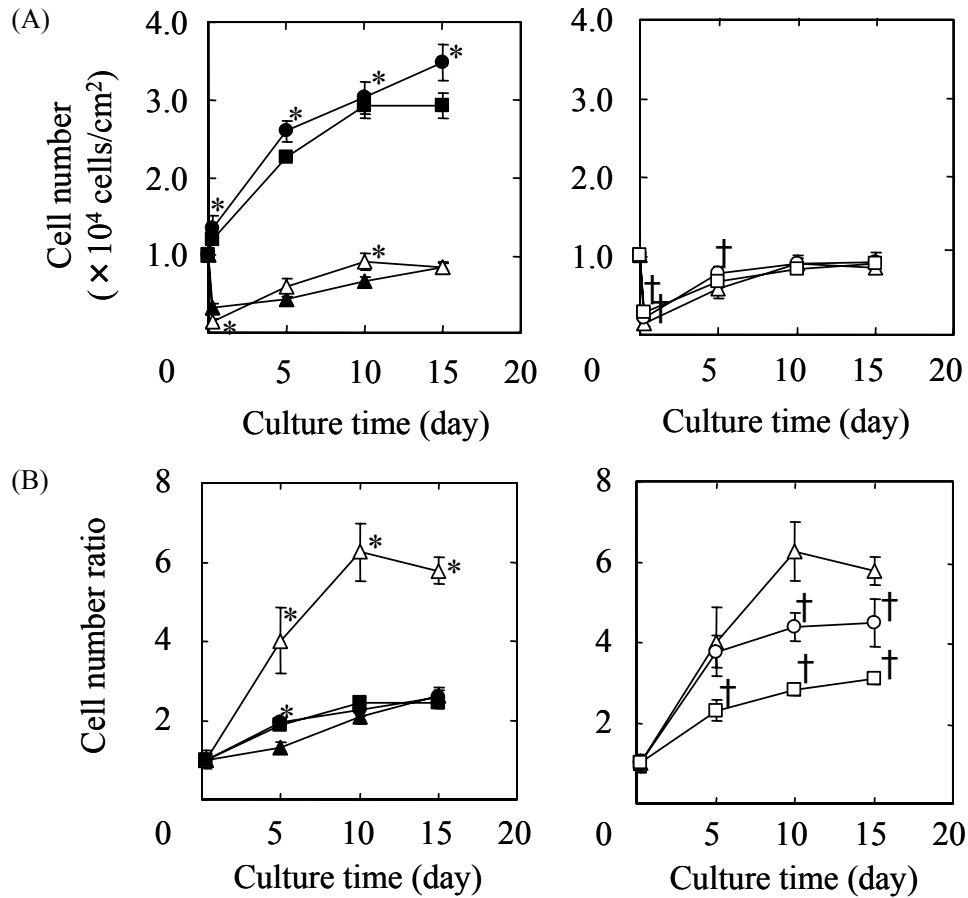
Figure 6. Time course of fat-derived stemcells proliferation after the static (●,▲, and ■) or agitated seeding and culture (○,△, and □). The cells were seeded on the PET films (●) or the PET non-woven fabrics before (▲ and △) and after NaOH treatment (○). RGD was immobilized on the fabrics NaOH treated following the pretreatment of EDC and NHS (□). As a control, cells were seeded and cultured on a cell culture dish (■). (A) The absolute number of cells proliferated.

*, $p < 0.05$; significant against the number of cells proliferated on PET non-woven fabrics after the static seeding.

†, $p < 0.05$; significant against the number of cells proliferated on PET non-woven fabrics after the agitated seeding.

(B) The number ratio of cells proliferated. The ratio was calculated regarding the number of cells after seeding as 1 for every sample.

*, $p < 0.05$; significant against the number ratio of cells proliferated on PET non-woven fabrics after the static seeding.

†, $p < 0.05$; significant against the number ratio of cells proliferated on PET non-woven fabrics after the agitated seeding.

FAK phosphorylation of cells cultured on two- or three-dimensional RGD-immobilized PET substrates by static and agitated culture methods

Figure 7 shows the phosphorylation of FAK for fat-derived stemcells cultured for 3 hr on various PET substrates. The FAK phosphorylation of cells tended to enhance for the PET films compared with the PET non-woven fabrics. The RGD immobilization on PET non-woven fabrics tended to increase FAK phosphorylation.
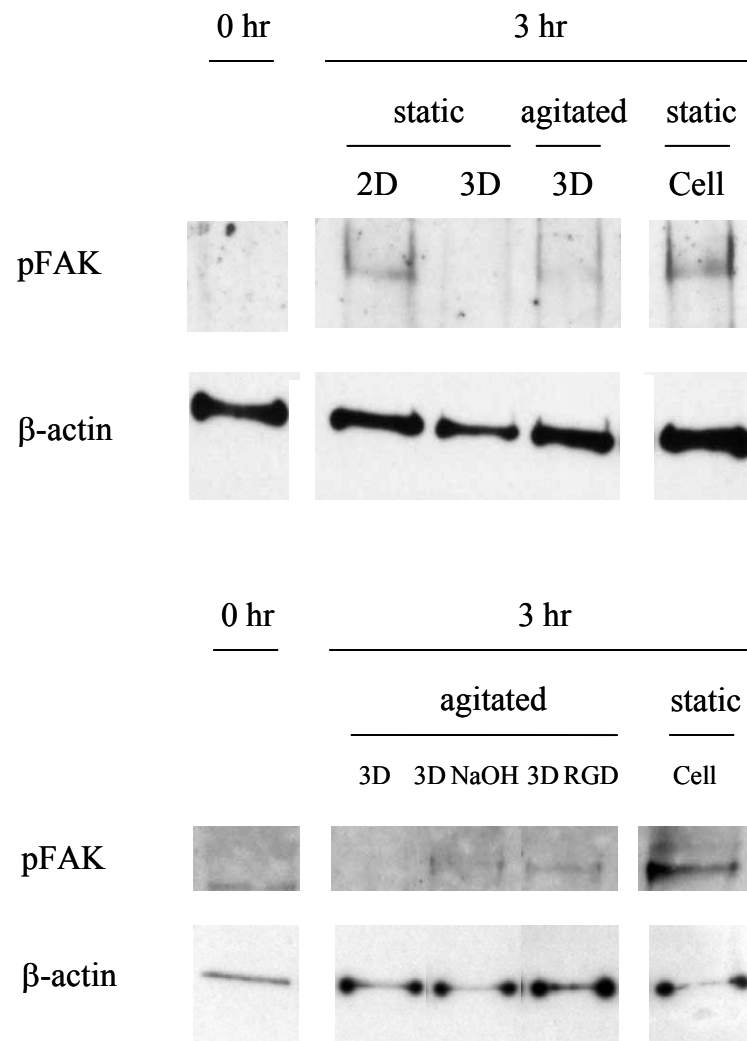
Figure 7. Phosphorylation of FAK in fat-derived stemcells on the PET substrates 3 hr after the static or agitated seeding. The cells were seeded on the PET film (2D) and the PET non-woven fabrics (3D), that NaOH treated (3D NaOH) or that NaOH treated and RGD immobilized (3D RGD). As a control, cells were seeded on a cell culture dish (Cell).

DISCUSSION

This Chapter investigates the adhesion and proliferation of human fat-derived stemcells for the PET films of 2D substrate and non-woven fabrics of 3D substrate.

Treatment of PET with NaOH aqueous solution increased the carboxyl groups for both the 2D and 3D PET substrates due to the generation caused by the chemical breakage of PET main chain. It is likely that the increased carboxyl groups made the surface more hydrophilic, resulting in decreased water contact angle (Figure 1 (A)). As apparent in Figure 1 (B), the carboxyl groups density of the PET films was in the level similar to that of the PET non-woven fabrics. The similar chemical reaction was performed for both the 2D and 3D substrates. The RGD density for the PET non-woven fabrics with pretreatment of EDC and NHS was higher than that of no pretreatment (Figure 2). It is possible that without pretreatment of EDC and NHS, the RGD was physically adsorbed on the PET non-woven fabrics. The significant increase of RGD density is due to chemical immobilization of RGD on the PET non-woven fabrics. The RGD density for the PET non-woven fabrics with NaOH treatment increased by 3.8-fold compared with those of no NaOH treatment. The increase level of RGD density was lower than that of carboxyl group. The carboxyl group density on NaOH-treated PET non-woven fabrics was 430 $pmol/cm^2$. The RGD density on the PET non-woven fabrics was 10 $pmol/cm^2$. Since the excessive amount of RGD was used for immobilization reaction, the difference in the increased level might be due to the steric hindrance effect of RGD molecules for immobilization. Yoon et al. showed that the density of 2-8 $pmol/cm^2$ affected cell adhesion [25]. Maheshwan et al. showed that the density of 0.17-17 $pmol/cm^2$ affected the cell migration, adhesion strength, and focal contact formation [26]. This indicates that the NaOH treatment generated the number of carboxyl groups on PET non-woven fabrics enough for RGD immobilization.

Fat-derived stemcells adhered onto the PET films similarly to the cell culture dish as a control (Figure 4). In general, the cell adhesion is affected by the water contact angle of substrate surface [21]. It is known that substrates with water contact angles of 60-80° are suitable for cell adhesion [27]. Since the water contact angle of PET films was 80°, PET is a substrate material suitable for the culture of fat-derived stemcells.

In many researches that the cell behavior was investigated for 2D and 3D substrates [13-15], the type of substrates, the cell density, the medium volume, and culture method were different. Therefore, it is practically impossible to directly compare the cell behavior. In this experiment for the static seeding method, the cell seeding density was adjusted to be similar by changing the concentration of cell suspension, considering the medium volume for the PET films (500 μl of cell suspension, 2 cm^2 of 24-well multiwell cell culture plate) and non-woven fabrics (75

μl of cell suspension, 0.3 cm^2 of 96-well multiwell cell culture plate). The cell number ratio adhered onto the PET non-woven fabrics was lower than that of the PET films. The difference in the cell adhesion can be explained by the following two reasons. The first reason is the possibility of cells to contact the material of substrates during the static seeding. All the cells necessarily contact the 2D substrate, whereas they do not necessarily contact the materials of 3D substrates because of the thinner fiber and porous structure. The second reason is due to the low affinity of 3D substrates for cell adhesion. The second reason may be more acceptable, because the agitated seeding method did not enhance the level of cell adhesion. From the FAK activation experiment, FAK phosphorylation tended to become higher for PET films than the PET non-woven fabrics. It is well known that the phosphorylation of FAK is related to the cell adhesion [28, 29]. This indicates that the 3D substrate gave cells the stimulus weaker than the 2D one. Hamilton et al. showed that the surface topography of substrates affected the FAK activation [30]. The surface modification increased the cell adhesion ratio of adipo-stromall cells seeded. It is possible that the NaOH treatment increases the carboxyl and hydroxyl group of substrates, resulting in enhanced protein adsorption onto the substrate and consequently promoted cell adhesion [31]. It is well recognized that the RGD motif interacts with the integrin on the membrane of cells, and then induces cell adhesion [12]. RGD immobilization further enhanced the cell adhesion. The phenomenon was also observed by the enhancement of FAK phosphorylation.

The proliferation of fat-derived stemcells was investigated at the same volume of medium per the surface area of PET substrates. The time profile of cell proliferation on the PET films was similar to that of the cell culture dish. This indicates that the PET was a material suitable for cell proliferation. For the static culture method, the absolute number of proliferated on the PET non-woven fabrics was lower than that of the PET films (Figure 6 (A)). This is because the film substrate has a large area which is suitable for cell proliferation than the non-woven fabrics. However, the time profile of cell number ratio was similar between the 2D and 3D substrates (Figure 6 (B)). This is not clear at present. From this finding, it is conceivable that the surface curvature dose not always affect the rate of cell proliferation. For the non-woven fabrics of 3D substrate, the agitated culture induced cell proliferation more effectively than the static one. It is possible that the agitation of culture medium gives cells more oxygen and nutrient, resulting in enhance cell proliferation. The similar time profile of cell proliferation was observed for the PET non-woven fabrics or those treated with NaOH and immobilized with RGD, whereas the cell number ratio was different. The difference can be explained in terms of the number of cells initially adhesion. Figure 6 shows that there was the maximum cell number to proliferate on the PET non-woven fabrics with or without NaOH treatment and RGD immobilization. The rate to reach the maximum cell number might be different because the number of cells initially adhesion was

different, resulting the difference of cell number ratio during the proliferation.

Fat-derived stemcells have their high potentials of proliferation and differentiation into osteogenic, adipogenic, chondrogenic, myogenic, and neurogneic cells [32-34]. It is expected that the potentials is useful to induce cells-based tissue regeneration. Therefore, it is necessary to develop the technology to allow the cells to effectively proliferate and differentiate. The agitated culture was suitable for 3D substrate from the viewpoint of cell proliferation. Considering that cells are always present in a 3D structure of body tissue, it is reasonable that the 3D substrate gives cells conditions closer to the natural environment. Further detailed investigation is needed to compare the cell behavior between the 2D and 3D substrates in terms of the culture system and the surface modification of substrates.

REFERENCES

1.   A. Hokugo, Y. Kubo, Y. Takahashi, A. Fukuda, K. Horiuchi, K. Mushimoto, S. Morita and Y. Tabata *Tissue Eng* **10**, 978 (2004)

2.   S. L. Ishaug-Riley, G. M. Crane-Kruger, M. J. Yaszemski and A. G. Mikos *Biomaterials* **19**, 1405 (1998)

3.   S. K. Moon, R. Park, H. Y. Lee, G. J. Nam, K. Cha, A. Andalibi and D. J. Lim *Acta Otolaryngol* **126**, 564 (2006)

4.   T. Ren, J. Ren, X. Jia and K. Pan *J Biomed Mater Res A* **74**, 562 (2005)

5.   Y. Takahashi and Y. Tabata *J Biomater Sci Polym Ed* **15**, 41 (2004)

6.   K. Yasuda, S. Inoue and Y. Tabata *Tissue Eng* **10**, 1587 (2004)

7.   S. Scaglione, A. Braccini, D. Wendt, C. Jaquiery, F. Beltrame, R. Quarto and I. Martin *Biotechnol Bioeng* **93**, 181 (2006)

8.   B. Inanc, A. E. Elcin and Y. M. Elcin *Tissue Eng* **12**, 257 (2006)

9.   T. Boontheekul, E. E. Hill, H. J. Kong and D. J. Mooney *Tissue Eng* **13**, 1431 (2007)

10.   Y. Ikada *Biomaterials* **15**, 725 (1994)

11.   Y. Li, T. Ma, S. T. Yang and D. A. Kniss *Biomaterials* **22**, 609 (2001)

12.   U. Hersel, C. Dahmen and H. Kessler *Biomaterials* **24**, 4385 (2003)

13.   A. Hadjizadeh, C. J. Doillon and P. Vermette *Biomacromolecules* **8**, 864 (2007)

14.   I. Amato, G. Ciapetti, S. Pagani, G. Marletta, C. Satriano, N. Baldini and D. Granchi *Biomaterials* **28**, 3668 (2007)

15.   T. G. Kim and T. G. Park *Tissue Eng* **12**, 221 (2006)

16.   Y. Hori, S. Inoue, Y. Hirano and Y. Tabata *Tissue Eng* **10**, 995 (2004)

17.   T. Richey, H. Iwata, H. Oowaki, E. Uchida, S. Matsuda and Y. Ikada *Biomaterials* **21**, 1057 (2000)

18.   N. Nakajima and Y. Ikada *Bioconjug Chem* **6**, 123 (1995)

19.   J. T. Smith, J. K. Tomfohr, M. C. Wells, T. P. Beebe, Jr., T. B. Kepler and W. M. Reichert *Langmuir* **20**, 8279 (2004)

20.   Y. Kimura, M. Ozeki, T. Inamoto and Y. Tabata *Tissue Eng* **8**, 603 (2002)

21.   S. Inoue, Y. Hori, Y. Hirano, T. Inamoto and Y. Tabata *J Biomater Sci Polym Ed* **16**, 57 (2005)

22.   R. M. Coleman, N. D. Case and R. E. Guldberg *Biomaterials* **28**, 2077 (2007)

23.   J. Rao and W. R. Otto *Anal Biochem* **207**, 186 (1992)

24.   M. Imamura, A. Kanematsu, S. Yamamoto, Y. Kimura, I. Kanatani, N. Ito, Y. Tabata and O. Ogawa *Am J Physiol Renal Physiol* **293**, F1007 (2007)

25. J. J. Yoon, S. H. Song, D. S. Lee and T. G. Park *Biomaterials* **25**, 5613 (2004)

26. G. Maheshwari, G. Brown, D. A. Lauffenburger, A. Wells and L. G. Griffith *J Cell Sci* **113 (Pt 10)**, 1677 (2000)

27. Y. Tamada and Y. Ikada *in: Polymer in Medicine II* p. 101. (1986)

28. P. J. Reddig and R. L. Juliano *Cancer Metastasis Rev* **24**, 425 (2005)

29. W. Guo and F. G. Giancotti *Nat Rev Mol Cell Biol* **5**, 816 (2004)

30. D. W. Hamilton and D. M. Brunette *Biomaterials* **28**, 1806 (2007)

31. N. Faucheux, R. Schweiss, K. Lutzow, C. Werner and T. Groth *Biomaterials* **25**, 2721 (2004)

32. P. A. Zuk, M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim and M. H. Hedrick *Mol Biol Cell* **13**, 4279 (2002)

33. P. A. Zuk, M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz and M. H. Hedrick *Tissue Eng* **7**, 211 (2001)

34. K. M. Safford, K. C. Hicok, S. D. Safford, Y. D. Halvorsen, W. O. Wilkison, J. M. Gimble and H. E. Rice *Biochem Biophys Res Commun* **294**, 371 (2002)

SUMMARY

Chapter 1

The objective of this study is to investigate the proliferation and differentiation of fat-derived stem cells derived from human fat tissues cultured on substrates with different surface properties. In addition, the similar investigation was performed for the cells proliferated in different concentrations of bFGF. The culture substrates include several polymer films with different water wettabilities, glass or a cell culture plate, and that coated with collagen type I or IV, gelatin, and bFGF. The proliferation profiles of cells were influenced by the type of culture substrates and the growth factor concentration. A larger number of cells proliferated was observed for substrates with the water wettability around 80° while the cell number was significantly larger for every protein-coated substrate. The rate of cell proliferation became maximum in a bFGF concentration of 1,000 ng/ml. The bFGF concentration used for cell proliferation affected the differentiation profile of cells proliferated. The fat-derived stem cells proliferated in 1 ng/ml bFGF were osteogenically differentiated to the strongest and fastest extent among those in other growth factor doses. The ALP activity of cells increased with the increased cell number although the activity per cells was indentical, irrespective of the substrates type. The strongest adipogenic differentiation was observed for cells proliferated in 1,000 ng/ml bFGF and the differentiation induction was maintained for a long time period. No clear dependence of the cell number on adipogenesis was observed. These findings indicate that the proliferation and differentiation of human fat-derived stem cells are influenced by the culture substrate and the concentration of bFGF used for proliferation.

Chapter 2

The objective of this study is to investigate the spreading area, proliferation, and adipogenic differentiation of fat-derived stem cells cultured on the surface of SAM prepared by alkanethiols with hydroxyl (OH), methyl (CH_3), amine (NH_2), and carboxyl terminal groups (COOH) or the mixture at different ratios. A modified enzyme-linked immunosorbent assay (ELISA) examination revealed that a high adsorption of vitronectin and fibronectin was observed for the SAM of NH_2 or the mixed SAM of OH and NH_2 groups, and the mixed SAM of OH and CH_3 or OH and COOH groups, respectively. The cell spreading area and the proliferation level were higher for the SAM of NH_2 or COOH or the mixed SAM of OH and NH_2 or OH and COOH groups than those of other substrates. When incubated in an adipogenic differentiation medium, the cells showed a high level of GPDH activity for the SAM of CH_3 or the mixed SAM of OH and COOH groups. In addition, a high mRNA expression of PPRAγ2 and aP2 was observed. For the SAM of NH_2 or COOH groups, the strong activation of ERK1/2 was observed while the mRNA expression of

connective tissue growth factor (CTGF) and cystein-rich 61 (CYR61) was enhanced. The proliferation of the cells was significantly suppressed by adding an inhibitor of ERK1/2. The mRNA expression of PPARγ2 was significantly induced by adding the inhibitor. It is concluded that the proliferation and adipogenic differentiation of fat-derived stem cells depended on the chemical composition of substrate surface, although the extent was influenced by that of ERK1/2 activation.

Chapter 3

Human fat-derived stem cells were incubated in the culture medium containing bFGF or on the cell culture dish coated with bFGF. The effect of the two existence modes of bFGF on the cell attachment and differentiation was evaluated. The morphology and number of cells were similar for both the bFGF modes. However, the ERK1/2 activation of cells incubated with bFGF in the medium was stronger and lasted for a longer time period than that of bFGF in the coated form. When fat-derived steml cells were incubated in an adipogenic differentiation medium, irrespective of the mode of bFGF added, the mRNA expression of PPRAγ2 and aP2, GPDH activity, and the cell accumulation of oil lipids were all suppressed. bFGF in the solution form inhibited the PPARγ2 expression to a high extent compared with that in the coated form. It is possible that bFGF-induced ERK1/2 activation consequently inhibited the adipogenic differentiation of fat-derived stem cells.

Chapter 4

The objective of this study is to investigate the adhesion of human fat-derived stem cells on the SAM with different surface densities and gradients of RGD peptide. The different densities and gradients of carboxyl groups on the SAM surface were prepared by a SAM exchange technique, and then RGD was chemically immobilized to allow the RGD surface density on the SAM to change over the range of 2.3-8.9 ng/cm^2 (3.0-12 pmol/cm^2). The spreading area and percent alive of cells increased as the density of RGD immobilized on the SAM became high, whereas no dependence of the RGD density on the number of cells adhered was observed. The percent alive of cells tended to increase with an increase in the RGD immobilization for the carboxyl group-gradient SAM. This finding suggests the possibility that the cell adhesion can be regulated by controlling the RGD density or gradient of cell substrate.

Chapter 5

The initial attachment, proliferation, and osteogenic differentiation of fat-derived stem cells from human fat tissue were investigated in three-dimensional non-woven fabrics prepared from PET fiber with different diameters. The largest number of cells initially attached was observed in the non-woven fabrics prepared from the PET fiber with a diameter of 22.0 μm, irrespective of the fabric

porosity. The number of cell attachment was larger and the cells were distributed more homogeneously in the fabrics by the agitated seeding method than by the static one. The culture method depended on the time profile of cell proliferation. The cell proliferation became better in order of the stirred (spinner flask) > the agitated > the static culture method. In addition, the cells were homogeneously proliferated in the fabrics by the stirred culture method. When evaluated as a measurement of cell osteogenic differentiation, the activity of ALP was not influenced by the diameter of fabrics. The static culture method tended to enable cells to enhance the ALP activity, in contrast with the stirred and agitated culture methods. It is concluded that the fabric fiber diameter and the culture method greatly affected the proliferation and differentiation of cells in the non-woven fabrics.

Chapter 6

The adhesion and proliferation of human fat-derived stem cells were investigated for the PET films of two-dimensional (2D) substrate and non-woven fabrics of three-dimensional (3D) substrate. When seeded by a static seeding method, more cells were adhered on the film than the non-woven fabric. However, the number ratio of cells proliferated to those initially adhered was similar. The cell proliferation was enhanced by an agitated culture method for the non-woven fabric. NaOH treatment introduced carboxyl groups into the surface of substrates, irrespective of the substrate type. A cell adhesion of peptide, RGD was chemically immobilized through the carboxyl groups at the density of 10 pmol/cm^2 on the non-woven fabric surface. The RGD immobilization significantly increased the number of cells adhesion after the agitated seeding method, but did not affect the cell proliferation. Phosphorylation of FAK was also enhanced by the RGD immobilization for the PET non-woven fabrics.

LIST OF PUBLICATIONS

Chapter 1

S. INOUE, Y. HORI, Y. HIRANO, T. INAMOTO, and Y. TABATA

Effect of culture substrate and fibroblast growth factor addition on the proliferation and differentiation of human fat-derived stem cells

J. Biomater. Sci. Polymer Edn., **16,** 57-77 (2005)

Chapter 2

S. INOUE, M. IMAMURA, A. UMEZAWA, and Y. TABATA

Attachment, proliferation, and adipogenic differentiation of adipo-stromal cells on self-assembled monolayers of different chemical compositions

J. Biomater. Sci. Polymer Edn., In press

Chapter 3

S. INOUE, M. IMAMURA, and Y. TABATA

Adipogenic differentiation of adipo-stromal cells incubated with basic fibroblast growth factor in the solution and coated form

J. Biomater. Sci. Polymer Edn., submitted

Chapter 4

S. INOUE, Y. IIDA, Y. OTANI, Y. HIRANO, and Y. TABATA

Adhesion behavior of human adipo-stromal cells on self-assembled monolayers with different surface densities or gradients of RGD peptide

J. Biomater. Sci. Polymer Edn., submitted

Chapter 5

K. YASUDA, S. INOUE, and Y. TABATA

Influence of culture method on the proliferation and osteogenic differentiation of human adipo stromal cells in non-woven fabrics

Tissue Eng., **10**, 1587-1596 (2004)

Chapter 6

S. INOUE, M. IMAMURA, Y. HIRANO, and Y. TABATA

Adhesion and proliferation of human adipo-stromal cells for poly(ethylene terephthalate) non-woven

fabrics with or without RGD immobilization

J. Biomater. Sci. Polymer Edn., submitted

Other publications

Y. HORI, S. INOUE, Y. HIRANO, and Y. TABATA

Additional effect of fibroblast growth factor on the proliferation and differentiation of rat mesenchymal stem cells on various substrates

Tissue Eng., **10**, 995-1005 (2004)

H. HOSSEINKHANI, Y. INATSUGU, Y. HIRAOKA, S. INOUE, H. SHIMOKAWA, and Y. TABATA

Impregnation of plasmid DNA into three-dimensional scaffolds and medium perfusion enhance in vitro DNA expression of mesenchymal stem cell

Tissue Eng., **11**, 1459-75 (2005)

H. HOSSEINKHANI, Y. INATSUGU, Y. HIRAOKA, S. INOUE, and Y. TABATA

Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly(glycolic Acid) fiber

Tissue Eng., **11**, 1476-88, (2005)

H. HOSSEINKHANI, M. YAMAMOTO, Y. INATSUGU, Y. HIRAOKA, S. INOUE, H. SHIMOKAWA, and Y. TABATA

Enhanced ectopic bone formation using a combination of plasmid DNA impregnation into 3-D scaffold and bioreactor perfusion culture

Biomaterials, **27**, 1387-1398 (2006)

K. HIROSE, A. MARUI, Y. ARAI, T. NOMURA, S. INOUE, K. KANEDA, T. KAMITANI, M. FUJITA, M. MITSUYAMA, Y. TABATA, and M. KOMEDA

Sustained-release vancomycin sheet may help to prevent prosthetic graft methicillin-resistant Staphylococcus aureus infection

Journal of Vascular Surgery, **44**, 377-382 (2006)

Y. SUN MOON, H. UYAMA, S. INOUE, and Y. TABATA

Fabrication of non-woven mats of gelatin/poly(L-lactic acid) composites by electrospinning and their

application for scaffold of cell proliferation

Chemistry Letters, **35**, 564-565 (2006)

S. INOUE and Y. TABATA

Influence of basic fibroblast growth factor in the solution and adsorbed form on the proliferation and differentiation of cells

Inflammation and Regeneration, **26**, 181-184 (2006)

Y. MANOME, T. KOBAYASHI, M. MORI, R. SUZUKI, N. FUNAMIZU, N. AKIYAMA,

S. INOUE, Y. TABATA, and M. WATANABE

Local delivery of doxorubicin for malignant glioma by a biodegradable PLGA polymer sheet.

Anticancer research, **26**, 3317-3326 (2006)

ACKNOWLEDGMENTS