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Studies on Distribution and Differentiation of Adipose Progenitor Cells in Rat Adipose Tissue

穴山 久志

2016年
Contents

Preface ........................................................................................................................................... 1

Chapter 1 Adipose Progenitor Cells Reside Among the Mature Adipocytes: Morphological Research Using an Organotypic Culture System

Section 1 Establishment of an Organotypic Culture System Using Adipose Tissue Slices of Rats

Introduction .................................................................................................................................... 9

Materials and Methods .................................................................................................................. 11

Results ........................................................................................................................................... 14

Discussion ...................................................................................................................................... 16

Summary ....................................................................................................................................... 18

Figure Legends ............................................................................................................................... 19

Figures ........................................................................................................................................... 21

Section 2 Localization of Adipose Progenitor Cells within Rat Adipose Tissue and Characterization of These Cells After the Adipogenic Stimulation in an Organotypic Culture System
Chapter 2 Histological and Immunohistochemical Analyses for the Differentiation of Adipose Progenitor Cells in Genetically Obese Wistar fatty rats

Section 1 Histopathological Evaluation of Adipose Tissue of Wistar Fatty Rats

Introduction .............................................................................................................49

Materials and Methods ..........................................................................................51

Results ......................................................................................................................53

Discussion ...............................................................................................................56

Summary .................................................................................................................59

Figure Legends ......................................................................................................61
Section 2  Identification of Adipose Progenitor Cells and its Differentiation in Adipose Tissue of Wistar Fatty Rats

Introduction ............................................................................................................................................. 66

Materials and Methods .......................................................................................................................... 68

Results .................................................................................................................................................. 71

Discussion ............................................................................................................................................ 73

Summary .............................................................................................................................................. 76

Figure Legends ..................................................................................................................................... 77

Figures ................................................................................................................................................ 78

Chapter 3  Effects of Lithium Chloride, a GSK-3β Inhibitor, on the Adipose Differentiation from Preadipocytes

Introduction ............................................................................................................................................ 81

Materials and Methods ....................................................................................................................... 83

Results .................................................................................................................................................. 87

Discussion ............................................................................................................................................ 91
Preface

The obesity is defined as an excessive fat accumulation in the body that correlates a risk of health. Body mass index (BMI) is a parameter for the definition of obesity, and the people who show BMI of $>30 \text{ kg/m}^2$ are considered to be obese in western countries. In United States, the population in obese is largely increased during last several decades, and the number of patients has been closed to 80 million (Ogden et al., 2014). The rate of obesity in Japan is lower than that of western countries; however, it is increasing especially in males, with a social problem in this country. While obesity is one of the component of metabolic diseases, this symptom plays a key role in the etiology of metabolic diseases, particularly atherosclerosis, hyperlipidemia, type II diabetes mellitus, and hypertension. In addition to the genetical risk, environmental factor, such as high energy intake, high fat food, or physiological inactivity, can be a cause of obesity (Dedoussis et al., 2007), and the primary treatment for obesity consists of refinement of diet and physical exercise. As for the medication, Orlistat (a pancreatic lipase inhibitor) and Lorcaserin (serotonin-2c receptor agonist) have been approved for the treatment of obesity; however, the classes of anti-obesity drugs are still limited. Therefore, it is an important research field for the development of novel drugs.

The obesity consists of histopathological hypertrophy of individual adipocytes and an increase in the number of adipocytes. Hypertrophy of the adipocytes, which is the result of
triglyceride accumulation in pre-existing adipocytes, precedes hyperplasia of the adipocytes during the development of obesity (Hausman et al., 2001). Although hyperplasia of the adipocytes is considered to be caused by the recruitment of new adipocytes from their progenitor cells, the details of the mode of differentiation from progenitor cells to mature adipocytes, through preadipocytes, are still under investigation.

As a part of investigation of adipose differentiation from preadipocytes, Hausman et al. reported several insights based on detailed morphological examinations using electron microscopy (Hausman and Martin, 1981; Hausman et al., 1980). Although there is a theory that adipose progenitor cells originate from outside the adipose depot, particularly from the bone marrow (Crossno et al., 2006), recent reports have been focusing on the existence of adipose progenitor cells within/beside the blood vessel walls (Gupta et al., 2012; Majesky et al., 2012; Tang W. et al., 2008; Tran et al., 2012; Zimmerlin et al., 2010). Tang et al. described that adipose progenitor cells reside in the mural cell component of the adipose vasculature (Tang W. et al., 2008), and Gupta et al. demonstrated that the endothelial cells and perivascular cells in mouse adipose tissue expressed Zfp423, a regulator gene of preadipocyte commitment (Gupta et al., 2012).

Over the past several decades, stem cell research became a great interest of biological science. Several innovative researches elucidated the mechanism of maintenance of
tissues/organs homeostasis via recruitment of stem cells, and regenerative therapy is attractive approach especially for unmet-medical needs. Therefore, discovery of the appropriate methods of cell/tissue implantation (cell therapy) and drugs that accelerate the regeneration of tissues/organs (regenerative medicines) is under the active competition in industries against the severe tissue losses associated with the diseases or injuries. According to the progress of stem cell research, regenerative therapy has expanded its target area and the utilization for the liver, kidney, heart, lung, neuronal tissue, or muscle is under the investigation.

Stem cells are classified as 2 categories that consist of artificially engineered stem cells and inherent stem cells. Embryonic stem cell (ES cell) and induced pluripotent stem cell (iPS cell) are generated by cell engineering technology and are considered to be candidates for cell therapy; however, the clinical application of these cells has not been accomplished due to the ethical issue and the risk of its tumorigenesis or immunogenicity leading to rejection response. On the other hand, inherent stem cells such as mesenchymal stem cells in the parenchymal tissues or in the bone marrow can be utilized for the autologous transplantation, and several clinical trials using mesenchymal stem cells are ongoing as the cell therapy for myocardial ischemia or others.

Many researches have revealed that the adipose tissue contains a large number of multipotent stem cells (Baer and Geiger, 2012; Zuk et al., 2002), and therefore the adipose
tissue is expected to be a promising source of progenitor cells for regenerative therapies (Qayyum et al., 2012). These are based on the results from investigations using the stromal vascular fraction (SVF), which is a heterogeneous cell mixture isolated by the enzymatic dissociation of adipose tissue, and adipose-derived stem cells (ASCs) contained in SVF are identified by cell surface markers such as CD34, CD90 or CD105 (Baer and Geiger, 2012; Cawthorn et al., 2012). The ASCs have been considered to be a major source for adipogenesis not only in healthy but also pathological conditions. Recently the localization of cells which are positive for these ASC markers in in vivo samples of adipose tissue has been demonstrated (Maumus et al., 2011; Zimmerlin et al., 2010); however, there might be a gap in the differentiation state of the mesenchymal cells or stem cells between in vivo samples and SVF after in vitro dissociation (Zeve et al., 2009). In addition, the extracellular matrix (ECM) has important functions for maintaining the cell morphology and cell fate of the adipocytes (Gattazzo et al., 2014; Huang and Greenspan, 2012; Mariman and Wang, 2010), and therefore, it was considered that the investigation of correlation between preadipocytes and ASCs within adipose tissue mass should be conducted in in vivo model or alternative model could keep the stemness of stem cells.

Recently, the obesity is considered to be one of the inflammatory disease (Revelo et al., 2014). This theory is based on the histopathological and clinical pathological features in obese
subjects. Nishimura et al. reported a histomorphological finding “crown-like structure” in visceral adipose tissue in ob/ob mice (Nishimura et al., 2007). The characteristic change has been confirmed in other animal model (Shaul et al., 2010) and human subjects (Bremer et al., 2011), and the structure consists of crown-like arrangement of macrophages surrounding individual adipocytes, adipocytes undergoing cell death, and the absence of perilipin immunoreactivity in dead adipocytes. In healthy condition, the white adipose tissue is composed of unilocular adipocytes tightly packed and surrounded by loose connective tissue including a network of capillaries. During the obesity, macrophage infiltration with forming crown-like structure appears in the white adipose tissue along with the hypertrophy and hyperplasia of adipocytes, and this initial cell infiltrate has crucial role for further inflammatory response by pro-inflammatory cytokines (Revelo et al., 2014). The trigger of macrophage infiltration is suspected to be a death of adipocytes; however, the mechanism of cell death is unclear. Thus, there are unexplored points in the biology of the white adipose tissue during the obesity, and in vivo animal model for the obesity must be useful tool for the investigation for this purpose because of the uniformity of histopathological changes with human.

In this thesis, a series of in vitro and in vivo studies were conducted to explore the origin of adipocytes (preadipocytes) and underling mechanism of adipogenesis. In Chapter 1, the author tried to establish the culture system of adipose tissue from normal rats for an
appropriate investigation system \textit{in vitro}. The procedures for the organotypic slice cultures of the central nervous system, in which the tissue slices were maintained on a Millicell-CM membrane for more than several months at the interface between the air and the culture medium (Stoppini et al., 1991; Tsuji et al., 2005), were modified for adipose tissue culture in the author’s experiment. As the results, the organotypic culture method was successfully established, and the investigation to elucidate the precise localization of adipose progenitor cells/preadipocytes and their characterization after the adipogenic stimulation was conducted using immunohistochemistry/immunocytochemistry methods with 3-dimmentional (3-D) observation by confocal laser microscope. Based on the insights derived from \textit{in vitro} assay in Chapter 1, the morphological/immunohistochemical characteristics and differentiation properties of preadipocytes in subcutaneous tissue of Wistar fatty rats, an animal model for the obesity with type II diabetes, was investigated in Chapter 2 to confirm the similarity of behavior of preadipocyte after the adipogenic stimulation \textit{in vitro} and in genetical obese condition \textit{in vivo}. Additionally, it has been reported that adipogenic differentiation of mesenchymal stem cells is negatively regulated by Wnt/\(\beta\)-catenin pathway (Tang Q. Q. and Lane, 2012). In Chapter 3, the effects of lithium chloride, a GSK-3\(\beta\) inhibitor, on the adipose differentiation from preadipocytes was evaluated using the organotypic culture system established in Chapter 1.
In conclusion, the present studies demonstrated that an organotypic culture system using adipose tissue slices was confirmed to be a useful tool for adipose tissue research. The results of these studies showed that adipose progenitor cells residing on mature adipocytes as well as the perivascular adipose progenitors were confirmed to be a source for adipogenesis, and that the transition of the differentiation stage of the preexisting mesenchymal cells to stem state was revealed to be involved in adipogenesis in rat adipose tissue in \textit{in vitro} and \textit{in vivo} systems. These are considered to be useful tools with information for the investigation of the mechanism of obesity and for the design of new anti-obesity drugs.
Chapter 1

Adipose Progenitor Cells Reside Among the Mature Adipocytes:
Morphological Research Using an Organotypic Culture System
Section 1

Establishment of an Organotypic Culture System Using Adipose Tissue Slices of Rats

Introduction

As for the progress of obesity, the hyperplasia of adipocytes is crucial phenomenon in addition to the hypertrophy of each adipocyte (Hausman et al., 2001). Despite the extensive studies have been conducted for several decades, the origin of progenitor cells and the detail of process of adipose differentiation from progenitor cells to mature adipocytes have not been fully cleared.

To investigate the morphological and biochemical transition of the progenitor cells or preadipocytes under various conditions of adipogenesis, research using the organotypic culture of adipose tissue is attractive; however, such culture method requires reproducibility of the in vivo architecture within the adipose tissue for accurate simulation. One organotypic culture method for adipose tissue has been reported (Sonoda et al., 2008). In their procedure, small pieces of rat adipose tissue were embedded within type-I collagen gel, and cultured successfully for 4 weeks. This method has been recognized as a useful in vitro system for investigation of the
adipocytes and mesenchymal stem-like cells. However, the vasculature within the adipose pieces disappeared shortly after the initiation of culture, and therefore, this culture method seems to be not suitable for the research considering the vasculature-adipocytes interaction. On the other hand, the organotypic culture methods using tissue slices have been developed for the central nervous system, and the nervous tissue slices could be maintained on a Millicell-CM membrane for more than several months at the interface between the air and the culture medium (Stoppini et al., 1991; Tsuji et al., 2005).

In this section, the author describes a simple organotypic culture method using adipose tissue slices as a tool for adipose tissue research.
Organotypic culture of adipose tissue slices

An outline of the organotypic culture is summarized in Fig. 1. The procedures for the organotypic slice cultures of the central nervous system, in which the tissue slices were maintained on a Millicell-CM membrane for more than several months at the interface between the air and the culture medium (Stoppini et al., 1991; Tsuji et al., 2005), were modified for adipose tissue culture in this experiment.

Six-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and, under deep anesthesia with isoflurane (Escain, Mylan Inc., Tokyo, Japan), the animals were euthanized by exsanguination from the abdominal aorta. The care and use of these animals and the experimental protocols for this study were approved by the Animal Experimental Use Committee of Takeda Pharmaceutical Company Limited (where the author conducted this study). The subcutaneous adipose tissue was removed from the inguinal region of the animals. Small pieces of adipose tissue were rinsed with DMEM-F12 medium (Gibco, Life Technologies Japan, Ltd., Tokyo, Japan), and then cut into 700-μm slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK). The tissue slices (2 to 4 slices/well) were put onto a membrane insert.
(Millicell-CM; Nihon Millipore K.K., Tokyo, Japan) and placed in a 6-well culture dish containing 1 mL of DMEM-F12 medium supplemented with 10 vol% newborn calf serum (Gibco, Life Technologies Japan, Ltd.) (10% NCS/DMEM). The slices were incubated at 37°C in a 5% CO₂ incubator and the culture medium was changed every 2 days.

**Morphological integrity of adipose tissue slices**

To confirm the histomorphology of the tissue slices, the adipose tissue slices before culture (Day 0) and on culture Days 1, 2, and 5 were fixed in 10 vol% neutral buffered formalin and embedded in paraffin. The entire slices were sectioned perpendicularly as shown in Fig. 1. Sections were stained with hematoxylin-eosin (H-E), and additional sections were stained immunohistochemically using anti-Ki-67 antibody (1:200; DAKO, Glostrup, Denmark; for cell proliferation) with an avidin-biotin complex (ABC) method using an ENVISION kit (DAKO) and diaminobenzidine (DAB) as a chromogen and counterstained with hematoxylin, and then examined under a light microscope.

Morphological observation of the H-E-stained sections from the slice cultures revealed that the tissue structure was well preserved in the core portion of the slices (details will be described in the results); however, spindle or polygonal cells were prominent at the bottom surface of the slice where the culture membrane was attached. Therefore, in the tissue
preparation in the following 3-D observations, all the tissue slices after the culture were also cut vertically to distinguish the changes in the core of the samples reflecting intended effects in the organotypic culture system from those at the bottom surface of the slice which could be a non-specific response in the cultured samples.

**Three-dimensional (3-D) observations using a confocal microscope**

*Fluorescent vital staining*

On culture Days 0, 1, 2, and 5, triple staining using lipid, endothelial cells and nucleic acid specific fluorescent dyes was performed on unfixed adipose tissues according to the previously described procedure (Nishimura et al., 2007; Suga et al., 2009). Briefly, the tissue slices were incubated with the following reagents for 30 min at 37°C: BODIPY 558/568 (5 μg/mL) for lipid staining of the adipocytes, Alexa Fluor 488-conjugated *Griffonia simplicifolia* isolecitin (GS-IB4, 20 μg/mL) for endothelial cells, and Hoechst 33342 (10 μg/mL) for the nuclei. All of these reagents were purchased from Molecular Probes (Eugene, Oregon, USA).

*Confocal microscope*

The 3-D structure and disposition of the adipose components were observed using a confocal microscope system (LSM700; Carl Zeiss Japan, Tokyo, Japan). The multistep observations were performed from the cut surface of the core portion of the tissue slices.
Results

Histological features after the organotypic culture of adipose tissue slices

Adipose tissue slices before culture (Day 0) were composed of numerous mature adipocytes containing large unilocular fat droplets with elongated nuclei and vessels including capillaries and small sized arteries/veins (Fig. 2A), and a small amount of connective tissue was also present. After the culture for up to 5 days, the tissue structure was well preserved even on culture Day 5 without significant degenerative changes and there were no obvious morphological changes in the adipose tissue components (Figs. 2B, 2C, 2D).

The 3-D observations of the adipose tissue components

Structural relationship of adipocytes, vasculature, and others

The 3-D observation of whole adipose tissue slices stained with 3 fluorescent dyes visualized the spatial configuration of the adipocytes and the capillary network. The capillary network extended throughout the adipose tissue mass and spread over the surface of the adipocytes (Pre-culture, Fig. 3A). No obvious changes in the adipose tissue components in the tissue slices after the 1-, 2- and 5-day cultures with the basic medium were confirmed by confocal microscopy as expected from the morphology in H-E-stained histology sections (Figs. 3B, 3C, 3D).
**Cell proliferation at the bottom surface of adipose tissue slices**

All of the above observations were related to the core portion of the adipose tissue slices and at the bottom surface, which was the surface attached to the culture membrane, the cellular organization was completely different. Spindle-shaped or polygonal cells were observed with higher density compared to the core portion of the tissue slices in culture (Fig. 4A), and a high proliferation rate of composing cells in contrast with core portion was confirmed by Ki-67 immunohistochemistry staining (Fig. 4B).
Discussion

A close relationship between the vascular system and adipogenesis has been reported (Baptista et al., 2009; Gupta et al., 2012; Lijnen, 2008; Nishimura et al., 2007; Tang W. et al., 2008; Tran et al., 2012; Zimmerlin et al., 2010). The author established the method of the tissue slice preparation using a tissue chopper and organotypic culture at the interface between the air and the culture medium for the subcutaneous adipose tissue from rats, because the organotypic slice cultures have the advantage of maintaining the physiological interactions between the cells and structural integrity and are widely used in brain research, although the method are also feasible for other research areas (Vidi et al., 2013). In the author’s system, the adipose tissue morphology not only in the adipocytes but also the vasculature, including arterioles and capillaries, was well retained in the slices up to culture Day 5. Thus, this method which maintains the vasculature seems to be suitable and provides further research capability for adipogenesis or obesity reflecting the vasculature-adipocytes interaction in terms of the investigation of the precise localization and biological characteristics of the adipose progenitor cells.

Meanwhile, marked expansion of spindle-shaped or polygonal cells was noted at the bottom surface of cultured adipose tissue slices. The cell proliferation which was confirmed by immunohistochemistry for Ki-67 was considered to be directly related to the culture method
using tissue slices. The bottom surface of the slices is attached to the culture membrane and closely exposed to the culture medium containing 10% NCS, and the cell proliferation might be a result of the mesenchymal cell reaction to the several growth factors in the culture medium. On the other hand, the extracellular matrix (ECM) regulates the proliferation activity of mesenchymal cells as well as cell differentiation (Gattazzo et al., 2014), and the stromal vascular cells separated by digestion of adipose tissue with collagenase show high proliferating activity on the cell culture plate (Hausman et al., 2008) and the proliferation of mesenchymal stem cell-like cells was observed at the peripheral part of the adipose tissue pieces after organotypic culture (Sonoda et al., 2008). Taken together, it was suggested that adipose mesenchymal cells, which presented under the suppressive control of the ECM within the tissue, expanded after the leaving a management of the ECM at the cut surface of the tissue slices and this phenomenon might relate to the extreme hyperplasia of adipocytes with the obesity.

In conclusion, an organotypic culture system using adipose tissue slices was confirmed to be a useful tool for adipose tissue research by morphological examinations.
Summary

The precise localization and biological characteristics of the adipose progenitor cells are still a focus of debate. In addition, to investigate the morphological and biochemical transition of the progenitor cells or preadipocytes under various conditions of adipogenesis, research using the organotypic culture of adipose tissue is attractive; however, such culture method requires reproducibility of the *in vivo* architecture within the adipose tissue for accurate simulation. In this study, simple organotypic culture system of adipose tissue slices was introduced and evaluated its usefulness as a platform for adipose research.

The tissue slices of subcutaneous white adipose tissue from rat were placed on a porous membrane and cultured at the interface between air and the culture medium for up to 5 days. The structure of adipose tissue components was sufficiently preserved during the culture without significant degenerative changes and there were no obvious morphological changes in the adipose tissue components. Histomorphological 3-D observation using confocal laser microscopy visualized the spatial configuration of the adipocytes and the capillary network.

Based on the above findings, the organotypic culture system using rat adipose tissue slices was confirmed to be a useful tool for adipose tissue research by morphological examinations.
Figure Legends

Fig. 1. The procedure of organotypic culture using adipose tissue slices. Subcutaneous adipose tissue of rats was sliced with a tissue chopper. Adipose tissue slices were placed on the porous membrane (Millicell-CM) and cultured at the interface between air and the culture medium. The lower picture indicates a representative whole image of a hematoxylin-eosin-stained adipose tissue slice after the 5 days culture (Scale bar = 200-μm).

Fig. 2. Histomorphology of adipose tissue slices (Core portion). H-E staining. Adipose tissue slices before culture were composed of mature adipocytes with a large lipid vacuole and a small flattened nucleus (arrows) uniformly (A). Following the culture, the tissue structure was well preserved even on culture Day 5 without significant degenerative changes (B-D). Scale bars = 25 μm.

Fig. 3. Fluorescent staining of whole adipose tissue slices. Before culture, the close connection between each adipocyte (red) and the capillary network (green) was confirmed (A). After the culture, no obvious changes in the adipose tissue components in the tissue slices after the 1- (B), 2- (C) and 5-day (D) cultures with the basic medium were confirmed. Scale bars = 50 μm.
Fig. 4. Histomorphology of cells at the bottom surface of adipose tissue slices on Day 5 culture.

H-E staining (A) and immunohistochemistry for Ki-67 (B, brown dye, arrows).

Spindle-shaped or polygonal cells were observed with higher density compared to the core portion at the bottom surface. These cells contained fine lipid droplets in their cytoplasm and showed high proliferation rate. Scale bars = 25 μm
Fig. 1
Fig. 2
Fig. 3

Fig. 4
Section 2

Localization of Adipose Progenitor Cells within Rat Adipose Tissue and Characterization of These Cells After the Adipogenic Stimulation in an Organotypic Culture System

Introduction

Regarding the origin of proliferating adipocytes in the obesity, extensive investigations have been conducted. These studies include electron microscopical examination of adipose tissue (Hausman and Martin, 1981; Hausman et al., 1980) or investigations on the possible origin of adipose progenitor cells from the bone marrow (Crossno et al., 2006). Recent reports have been focusing on the existence of adipose progenitor cells within/beside the blood vessel walls (Gupta et al., 2012; Majesky et al., 2012; Tang W. et al., 2008; Tran et al., 2012; Zimmerlin et al., 2010), and the stereoscopical examination is considered to be mandatory for the appropriate evaluation of relationship between adipose progenitor cells and somatic cells.

Many studies have revealed that the adipose tissue contains a large number of multipotent stem cells (Baer and Geiger, 2012; Zuk et al., 2002), and the adipose-derived stem cells have been considered to be a major source for adipogenesis not only in healthy but also
pathological conditions. In addition, the extracellular matrix (ECM) has important functions for maintaining the cell morphology and cell fate of the adipocytes (Gattazzo et al., 2014; Huang and Greenspan, 2012; Mariman and Wang, 2010), and the three-dimensional (3-D) culture system has been reported as desirable model to keep or improve the stemness of stem cells within the cultured tissues (Han et al., 2013; Shim et al., 2013).

To elucidate the precise localization of adipose progenitor cells/preadipocytes and their characterization after the adipogenic stimulation, the author utilize the organotypic culture method using rat adipose tissue slices, and detailed histomorphological observation was conducted.
Materials and Methods

Organotypic culture of adipose tissue slices

Six-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and, under deep anesthesia with isoflurane (Escaín, Mylan Inc., Tokyo, Japan), the animals were euthanized by exsanguination from the abdominal aorta. The care and use of these animals and the experimental protocols for this study were approved by the Animal Experimental Use Committee of Takeda Pharmaceutical Company Limited (where the author conducted this study). The subcutaneous adipose tissue was removed from the inguinal region of the animals. Small pieces of adipose tissue were rinsed with DMEM-F12 medium (Gibco, Life Technologies Japan, Ltd., Tokyo, Japan), and then cut into 700-μm slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK). The tissue slices (2 to 4 slices/well) were put onto a membrane insert (Millicell-CM; Nihon Millipore K.K., Tokyo, Japan) and placed in a 6-well culture dish containing 1 mL of DMEM-F12 medium supplemented with 10 vol% newborn calf serum (Gibco, Life Technologies Japan, Ltd.) (10% NCS/DMEM). The slices were incubated at 37°C in a 5% CO₂ incubator and the culture medium was changed every 2 days.

Adipogenic culture of adipose tissue slices
To examine the effects of the adipogenic factors, the adipose tissue slices were incubated with 10% NCS/DMEM supplemented with 5 μg/mL insulin (Sigma-Aldrich Japan K.K., Tokyo, Japan), 0.01 μg/mL dexamethasone (DEX; Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 2.2 μg/mL 3-isobutyl-1-methylxanthine (IBMX; Wako Pure Chemical Industries, Ltd.) during the initial 2 days and then the medium was changed to 10% NCS/DMEM supplemented with insulin. This mixture has been reported as a well-defined adipogenic cocktail in the studies using the 3T3-L1 preadipocyte cell line (Ntambi and Young-Cheul, 2000).

Morphological integrity of adipose tissue slices

To confirm the histomorphology of the tissue slices, the adipose tissue slices before culture (Day 0) and on culture Days 1, 2, and 5 were fixed in 10 vol% neutral buffered formalin and embedded in paraffin. The entire slices were sectioned perpendicularly. Sections were stained with hematoxylin-eosin (H-E), and additional sections were stained immunohistochemically using anti-Ki-67 antibody (1:200; DAKO, Glostrup, Denmark; for proliferating activity) with an avidin-biotin complex (ABC) method using an ENVISION kit (DAKO) and diaminobenzidine (DAB) as a chromogen and counterstained with hematoxylin, and then examined under a light microscope.
Three-dimensional (3-D) observations using a confocal microscope

**Fluorescent vital staining**

On culture Days 0, 1, 2, and 5, triple staining using lipid, endothelial cells and nucleic acid specific fluorescent dyes was performed on unfixed adipose tissues according to the previously described procedure (Nishimura et al., 2007; Suga et al., 2009). Briefly, the tissue slices were incubated with the following reagents for 30 min at 37°C: BODIPY 558/568 (5 μg/mL) for lipid staining of the adipocytes, Alexa Fluor 488-conjugated *Griffonia simplicifolia* isoelectin (GS-IB4, 20 μg/mL) for endothelial cells, and Hoechst 33342 (10 μg/mL) for the nuclei. All of these reagents were purchased from Molecular Probes (Eugene, Oregon, USA).

**Whole-mount immunohistochemistry**

Whole-mount immunohistochemistry was performed on culture Days 0, 1 and 2. The adipose tissue slices were fixed overnight with 4 w/v% paraformaldehyde (4% PFA) at 4°C and rinsed in phosphate-buffered saline (PBS). The tissue slices were pre-incubated with proteinase K (DAKO), methanol (Wako Pure Chemical Industries, Ltd.), and Tris buffered saline with Tween 20 (TBST; DAKO) sequentially. Samples were incubated with following primary antibodies: anti-CCAAT-enhancer-binding protein-β (C/EBP-β; 1:50; Bioworld Technology, Inc., Minnesota, USA), anti-fibronectin (1:100; DAKO), anti-α5 integrin (1:50; abcam, Tokyo,
Japan), or anti-CD105 (endoglin; 1:50; abcam) for 1 h at room temperature. After the incubation, following washing with TBST, the samples were reacted with a fluorochrome-conjugated secondary antibody (goat anti-rabbit Alexa Fluor 594 or goat anti-rabbit Alexa Fluor 488, 1:200; Molecular Probes) for 30 min at room temperature. Neutral lipids within the 4% PFA-fixed tissue slices were detected by a HCS Lipid TOX neutral lipid stain (Molecular Probes), and the endothelial cells and nuclei were stained with Alexa Fluor 594 conjugated GS-IB₄ (20 μg/mL; Molecular Probes) and 4,6-diamidino-2-phenylindole (DAPI; 1:000; Molecular Probes), respectively.

**Confocal microscope**

The 3-D structure and disposition of the adipose components were observed using a confocal microscope system (LSM700; Carl Zeiss Japan, Tokyo, Japan). The multistep observations were performed from the cut surface of the core portion of the tissue slices.
Results

Histological features after the organotypic culture of adipose tissue slices with adipogenic stimulation

As mentioned in section 1, adipose tissue slices before culture (Day 0) were composed of numerous mature adipocytes containing large unilocular fat droplets with elongated nuclei and vessels including capillaries and small sized arteries/veins (Fig. 1A), and some connective tissue was also present. With adipogenic stimulation with supplementation of a mixture of insulin, DEX, and IBMX, small-sized multilocular adipocytes appeared between the unilocular-mature adipocytes and/or perivascular spaces in the tissue slices on Day 1 or Day 2 (Figs. 1B, 1C). Those newly formed multilocular cells after adipogenic stimulation were polygonal to spindle-shaped and had fine cytoplasmic vacuoles and round nuclei. The formation of the multilocular cells progressed further under the influence of insulin by culture Day 5 and the number of multilocular cells and the size of cytoplasmic vacuoles increased time-dependently (Fig. 1D). There were no apparent morphological changes in the mature adipocytes.

Proliferative potential of the unilocular and multilocular adipocytes

In the pre-culture slices, the immunohistochemistry of Ki-67 showed that there was no
positive reaction in any of the adipocytes (Fig. 1E). In the samples with adipogenic stimulation, the adipocytes did not show active proliferative properties of neither the unilocular nor the multilocular type (Fig. 1F, Day 2).

The 3-D observations of the adipose tissue components

Structural relationship of adipocytes, vasculature, and others

In the tissue slices before culture, the mature adipocytes had oval nuclei and a large single spherical lipid droplet (Fig. 2A). The 3-D observations also confirmed the presence of several small cells, which had little or no fat droplets, in the perivascular regions or on the surfaces of the mature adipocytes (Fig. 2A, inset). These interstitial cells were clearly distinguishable from mature adipocytes or capillary endothelial cells by detailed observation at the serial Z position with a confocal microscope, and the number of cells located and perched on the surface of the mature adipocytes without any relationship to the capillaries.

Effects of adipogenic stimulation on the morphology of adipose tissue slices

The 3-D observations of the tissue slices after adipogenic stimulation revealed developing fine lipid droplets in the interstitial cells in the perivascular regions or on the surface of the mature adipocytes on Day 1 (Fig. 2B). These cells were transformed to multilocular adipocytes having more large lipid vacuoles after a 2-day adipogenic culture (Fig. 2C), and
numerous multilocular adipocytes containing prominent lipid droplets of various size were present between the mature adipocytes on culture Day 5 (data not shown).

*Evaluation of adipose differentiation of interstitial cells: immunohistochemistry for C/EBP-β*

For the characterization of adipogenetic transformation in the interstitial cells, expression of C/EBP-β, which is known to be induced in the early stage of adipocyte differentiation (Farmer, 2005; Hung et al., 2004; Lee K. et al., 1998), was investigated.

C/EBP-β expression was scarcely found in the nuclei of the interstitial cells in the adipose tissue on Day 0 and Day 2 with basic medium; however, C/EBP-β positive cells were abundantly increased between mature adipocytes after a 2-day adipogenic culture (Figs. 2D-G) along with an increase in the number of multilocular adipocytes.

*Distribution of fibronectin and α5 integrin in the adipose tissue and its relationship with interstitial multilocular adipocytes*

For the further characterization of adipogenic interstitial cells in the tissue slices, the relationship with fibronectin, a member of ECM and α5 integrin which is responsible as a cellular receptor of fibronectin was investigated. In the pre-culture tissue, the presence of fibronectin supporting mature adipocytes and vasculature was confirmed (Fig. 3A). Several interstitial cells located in the perivascular region were loosely encapsulated in
fibronectin-positive ECM. The adipose tissue slices after 2-days culture with basic medium showed the same features. Following the 2-days adipogenic culture, interstitial cells containing fine or small lipid droplets were found within the bundles of fibronectin (Fig. 3B). On the other hand, the α5 integrin immunoreactivity was found in only a limited number of endothelial cells in pre-culture and there were no positive reactions in the interstitial cells in both the pre-culture and adipogenic-cultured slices (Figs. 3C, 3D).

**Immunohistochemistry for CD105; detection of mesenchymal stem cells or cells at stem state**

To evaluate the possible changes in the cell-differentiation state during adipogenesis of the interstitial cells, the immunohistochemistry for CD105 was performed on the pre- and cultured slices. CD105 is known to be expressed in endothelial cells and, to confirm its relationship with the vasculature, the endothelial cells in the adipose tissue slices were visualized by isolectin after the immunohistochemistry for CD105 (Figs. 4A, 4B). After the organotypic culture, CD105 expression was up-regulated in the endothelial cells; however, there were no remarkable differences in the CD105 expression of the endothelial cells between the basic culture and the adipogenic culture by Day 2. There were only limited increases in the immunoreactivity in the interstitium in the Day 1 cultures (data not shown), and CD105-positive non-endothelial cells appeared in the tissue slices with 2-day adipogenic stimulation. For detailed localization of CD105-positive cells, some of the positive cells were present very
closely beside the capillary network, but many of the cells were present in the interstitial space independent of the vasculature.
For adipogenic stimulation using the organotypic culture, the adipogenic mixture containing insulin, DEX and IBMX, of which cocktail has been commonly used as an adipogenic stimulator in several in vitro systems using 3T3-L1 preadipocytes or stromal-vascular cells (Armani et al., 2010; Miyazaki et al., 2005; Ntambi and Young-Cheul, 2000), was used in this study and induced numerous multilocular adipocytes in the adipose tissue slices. In parallel with the appearance of multilocular adipocytes, there was an increase in the number of C/EBP-β expressing cells between the mature adipocytes. The transition of gene expression during adipogenesis has been well-investigated using an in vitro system of 3T3-L1 preadipocytes (Ntambi and Young-Cheul, 2000). A transcription factor, C/EBP-β is known to be induced in the early stage of adipocyte differentiation (Farmer, 2005; Hung et al., 2004; Lee K. et al., 1998). Therefore, it was considered that multilocular adipocytes were induced at the interstitium of the adipose tissue slices via adipogenic differentiation from progenitor cells.

The presence and localization of such progenitor cells are difficult to see in routine paraffin-embedded sections of the adipose tissue before culture; however, the 3-D observation using confocal laser microscopy on the whole adipose tissue slices revealed the spatial arrangement and the presence of such progenitor cells between the mature adipocytes. In addition, careful 3-D observation indicated the possibility of 2 populations of multilocular adipocytes.
adipocytes based on the localization in the adipose tissue slices. Multilocular adipocytes appearing at or beside the walls of the vasculature were one of those and are considered to originate from adipose progenitor cells which have been suggested to be localized at the vasculature by other authors (Gupta et al., 2012; Tang W. et al., 2008; Tran et al., 2012; Zimmerlin et al., 2010). Another population of multilocular adipocytes was formed on the mature adipocytes and these have no structural correlation with any part of the vasculature. Several reports have postulated that mature unilocular adipocytes can proliferate and divide during adipocyte proliferation (hyperplasia) to obesity (Nagayama et al., 2007; Sugihara et al., 1987). However, the author’s observations suggest the possibility that some of the newly-formed adipocytes in the obesity might differentiate from the adipose progenitor cells residing on the mature adipocytes and, in that case, the increased number of adipocytes is not fully dependent on true hyperplasia of the adipocytes (cell division of the adipocytes) but results from adipocytic differentiation of progenitor cells residing on the mature adipocytes. This hypothesis would be supported by the fact that no mitotic activity was observed in the polygonal multilocular adipocytes that appeared between the mature adipocytes following the adipogenic cultures in this study.

The progenitor cells of these multilocular adipocytes were embedded in the fibronectin-positive ECM supporting mature adipocytes. The cell fate including differentiation...
of the mesenchymal cells is influenced by the interaction with the ECM surrounding the cells (Gattazzo et al., 2014; Guilak et al., 2009). In the in vitro investigation using cell lines, it has been confirmed that the interaction between fibronectin, a major component of adipose tissue ECM, and α5 integrin for cellular receptor of fibronectin suppresses the adipogenic differentiation of preadipocytes (Nie and Sage, 2009), and switching of the ECM from fibronectin to laminin occurs at the onset of adipogenesis accompanied by increased expression of α6 integrin which is the cellular receptor for ECM laminin (Liu et al., 2005). The expression of α5 integrin in the adipose progenitor cells within the ECM of the tissue slices was not detected in the authors system and the alteration in α5 integrin/fibronectin mediated maintenance of the progenitor cells was not apparent; however, additional investigations for the expression of the ECM and cellular adhesion molecules may reveal the precise mechanism and control of the differentiation of adipose progenitor cells in the tissue.

Several studies on adipose-derived stem cells revealed that adipose tissue is a rich source of multipotent stem cells that show a fibroblast-like morphological appearance (Fernyhough et al., 2008; Gupta et al., 2012; Schaffler and Buchler, 2007). CD105, which is also referred to as endoglin, is generally recognized as a cell surface marker of mesenchymal stem cells or hematopoietic stem cells (De Schauwer et al., 2011). Recent reports showed that CD105 expression is fully detected in undifferentiated mesenchymal cells and is lost in
differentiated adipocytes during adipose differentiation (Mohsen-Kanson et al., 2013) and that CD105 expression would be related to the capacity of adipose differentiation (Zych et al., 2014). Thus, CD105 is considered as an important marker of undifferentiated mesenchymal cells having the potential of adipose progenitor cells. In the immunohistochemistry for CD105, only a limited number of endothelial cells of the capillaries were positively stained in the pre-culture slices. The organotypic culture in the author’s system demonstrated remarkable up-regulation of CD105 expression in the cells composing the adipose tissues while perivascular cells or interstitial cells on mature adipocytes showed transition to CD105-positive cells after adipogenic culture, suggesting acquisition of stem state or de-differentiation of these cells.

The several studies using nervous or skin tissue have elucidated that the 3-D culture system preserves the stemness/stem state of stem cells and further enhances the stemness or reprogramming of cells in cultured tissues (Han et al., 2013; Shim et al., 2013). Furthermore, the presence of adipose stem cells is suggested to participate in adipogenesis (Cawthorn et al., 2012; Kim et al., 2014). In the author’s system, several multilocular adipocytes appeared in the course of a day with adipogenic stimulation and these were considered to be differentiated from the pre-existing committed preadipocytes. The number of multilocular adipocytes expanded through the 2- and 5 days adipogenic treatments along with a remarkable increase in CD105 positive cells in the interstitial tissue. Therefore, in combination with the other observations
with morphology or C/EBP-β immunohistochemistry, the CD105-positive cells which were trans-differentiated/de-differentiated to the stem state from the preexisting state within the adipose tissue strongly suggested that these are a source of adipose progenitor cells in the adipogenic tissue culture. *In vivo* investigations using the adipose tissue of obese subjects will reveal this more clearly.

In conclusion, the results of this study showed that adipose progenitor cells residing on mature adipocytes as well as the perivascular adipose progenitors were confirmed to be a source for adipogenesis and the transition of the differentiation stage of the preexisting mesenchymal cells to stem state was revealed to be involved in adipogenesis in rat adipose tissue.
Summary

To elucidate the precise localization of adipose progenitor cells/preadipocytes and their characterization after the adipogenic stimulation, the author utilize the organotypic culture method using adipose tissue slices of rats, and detailed histomorphological observation was conducted. The tissue slices of subcutaneous white adipose tissue from rat were placed on a porous membrane and cultured at the interface between air and the culture medium for up to 5 days with or without adipogenic stimulation. Following adipogenic stimulation with insulin, dexamethasone, and 3-isobutyl-1-methylxanthine, numerous multilocular adipocytes appeared in the interstitium among the mature adipocytes. Histomorphological 3-D observation using confocal laser microscopy revealed the presence of small mesenchymal cells containing little or no fat residing in the perivascular region and on the mature adipocytes and differentiation from the preexisting mesenchymal cells to multilocular adipocytes. Immunohistochemistry demonstrated that these cells were initially present within the fibronectin-positive extracellular matrix (ECM). The adipose differentiation of the mesenchymal cells was confirmed by the enhanced expression of C/EBP-β suggesting adipose differentiation and the concurrent advent of CD105-expressing mesenchymal cells within the interstitium of the mature adipocytes. Based on the above, the mesenchymal cells embedded in the ECM around the mature adipocytes were confirmed to be a major source for adipogenesis while the transition to the stem state in the
mesenchymal cells was considered to contribute to the increase in the number of adipocytes in rat adipose tissue.
Figure Legends

Fig. 1. Effects of adipogenic stimulation on the histomorphology of adipose tissue slices (Core portion). H-E staining (A-D) and immunohistochemistry of Ki-67 (E and F, brown dye). Adipose tissue slices before culture were composed of mature adipocytes with a large lipid vacuole and a small flattened nucleus uniformly (A). Following the adipogenic stimulation, polygonal multilocular adipocytes appeared between mature adipocytes. These cells had small vacuoles and round nuclei, and the number of polygonal cells increased with the culture period through Day 1 (B), Day 2 (C), and Day 5 (D). Endothelial cells in the adipose tissue were positively stained with Ki-67 scarcely (E); however, multilocular adipocytes did not show active proliferative properties (F) Scale bars = 25 μm.

Fig. 2. Fluorescent staining of whole adipose tissue slices (A-C) and immunohistochemistry of C/EBP-β (D-G). Before culture, the close connection between each adipocyte (red) and the capillary network (green) was confirmed (A), and cells which had no lipid droplets were found on the surface of the mature adipocytes (A, inset). After the adipogenic culture, numerous multilocular cells having very small lipid droplets appeared on the surface of the mature adipocytes or beside the vasculature since Day 1 (B), and the lipid droplets of these cells became larger with a further culture period (C). Along with the
increase in the number of multilocular adipocytes, C/EBP-β-positive cells (indicated by red fluorescence in the nucleus) were increased with adipogenic culture (D-G, Day 2).

Scale bars = 50 μm.

Fig. 3. Immunohistochemistry for fibronectin (A and B) and α5 integrin (C and D). Several interstitial cells were found to be within the bundle of fibronectin-positive matrixes (red) in the adipose tissue slices before culture (A), and on Day 2 of adipogenic culture, adipocytes containing smaller lipid droplets (green) appeared within the fibronectin-positive tissues (B). The expression of α5 integrin, a cellular receptor for fibronectin, was not detected in the interstitial cells of before (C) or after the 2-days adipogenic culture (D). Scale bars = 50 μm.

Fig. 4. Immunohistochemistry for CD105, a cell surface marker of mesenchymal stem cells. In the pre-culture slices (A), positive reaction was not remarkable. On Day 2 of adipogenic culture (B), CD105 expression was up-regulated in the several cells beside the capillary network or interstitial cells that were present independently from the vasculature. Scale bars = 50 μm.
Fig. 2
Fig. 3
Fig. 4
Chapter 2

Histological and Immunohistochemical Analyses for the
Differentiation of Adipose Progenitor Cells in Genetically Obese
Wistar fatty rats
Section 1

Histopathological Evaluation of Adipose Tissue of Wistar Fatty Rats

Introduction

Over the last several decades, the obesity has become one of the most serious disorders in Western countries and several million people die each year as a result of the obesity and its-related diseases.

According to studies on adipose tissue biology, inflammation is considered important for the homeostasis of adipose tissue and adipocyte proliferation resulting in the obesity is associated with the dysregulation of the immune system in the adipose depots (Kohlgruber and Lynch, 2015; Revelo et al., 2014). The key players involved in adipose tissue immune response include mainly macrophages, as well as regulatory T-cells, granulocytes, and/or mast cells (Apostolopoulos et al., 2015), and these cells produce a variety of factors that could modify the adipocyte function and adipose tissue environment. Adipose tissue macrophages are generally polarized to the M2 phenotype under the non-obese conditions; however, in obesity, the balance shifts from the M2 to the M1 phenotype that is an activated and pro-inflammatory cell type (Kohlgruber and Lynch, 2015). Although hypoxia, adipocyte stress, or possible Toll-like
receptor activation are suggested as causes of adipocyte proliferation and disease progression (Guglielmi et al., 2015; Hong et al., 2015), the pathophysiology of adipose tissue-composing cells during the disease progression has not been fully understood.

There are several useful animal models for the obesity and its-related diseases, and the character identification of these models has great value for expanding the understanding of the biology underlying a state of health or disease (Lijnen, 2011). The Wistar fatty rat has been developed as an animal model of obesity-related, non-insulin-dependent diabetes mellitus by crosses between obese Zucker and Wistar-Kyoto rats (Ikeda et al., 1981; Yamakawa et al., 1995). The Wistar fatty rats show severe obesity, hyperinsulinemia, and hyperlipidemia, and this strain of animal has been well used in nonclinical pharmacology studies as a model of obesity and type 2 diabetes (Hayakawa et al., 1996; Ikezawa et al., 2005; Suzuki et al., 2002). However, the histopathological evaluation of the adipose tissue in Wistar fatty rats has not been conducted.

In this section 1, the author conducted detailed histomorphological examinations of the adipose tissue of Wistar fatty rats, an obese/diabetic model, using immunohistochemistry. Furthermore, the histomorphological results from Wistar fatty rats were compared with the findings reported in human obese subjects to explore the usefulness of this strain of rat as a disease model. In addition, confirming the findings obtained in Chapter 1 using the organotypic culture system focusing on the adipocyte differentiation is also a purpose of this Chapter 2.
Materials and Methods

Animals

Forty-nine-week-old male Wistar fatty (obese/diabetic) and Wistar lean (control) rats were purchased from Takeda Rabics Ltd. (Kanagawa, Japan) and, under deep anesthesia with isoflurane (Escain, Mylan Inc., Tokyo, Japan), the animals were euthanized by exsanguination from the abdominal aorta/posterior vena cava. The care and use of these animals and the experimental protocols for this study were approved by the Animal Experimental Use Committee of Takeda Pharmaceutical Company Limited (where the author conducted this study).

Morphological evaluation of adipose tissue of Wistar fatty rats

The subcutaneous adipose tissue was removed from the inguinal region of the animals. To confirm the histomorphology, parts of the adipose tissues were fixed in 10 vol% neutral buffered formalin, embedded in paraffin, and sectioned at approximately 4-μm thickness. The sections were stained with hematoxylin-eosin (H-E) and the additional sections were stained immunohistochemically using anti-Ki-67 antibody (1:200; DAKO, Glostrup, Denmark; for proliferating activity), anti-CD68 antibody (1:1000; Millipore, California, USA), and anti-CD11b antibody (1:2000, Novus Biologicals, Colorado, USA) with an avidin-biotin
complex (ABC) method using an ENVISION kit (DAKO) or Histofine Simple Stain MAX PO (R) (Nichirei Biosciences, Tokyo, Japan) and diaminobenzidine (DAB) as a chromogen and counterstained with hematoxylin, and then examined under a light microscope.

**Three-dimensional (3-D) observations using a confocal microscope**

After removal from the animals, small pieces of adipose tissue were cut into 700-μm slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK). The adipose tissue slices were fixed overnight with 4 w/v% paraformaldehyde (4% PFA) at 4°C, rinsed in phosphate-buffered saline (PBS) and were pre-incubated with proteinase K (DAKO), methanol (Wako Pure Chemical Industries, Ltd.), and Tris buffered saline with Tween 20 (TBST; DAKO) sequentially. Samples were incubated with the following primary antibodies: anti-fibronectin (1:100; DAKO) and anti-vimentin (1:50; DAKO) for 1 h at room temperature. After the incubation, and after washing with TBST, the samples were reacted with a fluorochrome-conjugated secondary antibody (goat anti-rabbit Alexa Fluor 594 or goat anti-rabbit Alexa Fluor 488, 1:200; Molecular Probes) for 30 min at room temperature. Neutral lipids within the 4% PFA-fixed tissue slices were detected by a HCS Lipid TOX neutral lipid stain (Molecular Probes), and the endothelial cells and nuclei were stained with Alexa Fluor 594 conjugated GS-IB4 (20 μg/mL; Molecular Probes) and DAPI, respectively. The 3-D structure and disposition of the adipose components were observed using a confocal microscope system.
Results

Histological features of subcutaneous adipose tissue of Wistar lean rats and obese/diabetic Wistar fatty rats

The adipose tissue of Wistar lean rats was composed mostly of adipocytes containing large unilocular fat droplets with elongated nuclei and limited visible cytosol, and the tissues were sometimes separated by thin connective tissues into the various sizes of lobules (Figs. 1A, 1B). Vessels including capillaries and small arteries/veins were also present. There were no notable inflammatory changes or necrotic/degenerative changes of adipose tissue in any animals.

Within the adipose tissue of obese/diabetic Wistar fatty rats, hypertrophy of each unilocular adipocyte was remarkable in all animals examined (Fig. 1C). These hypertrophic adipocytes contained single elongated nuclei and limited visible cytosol, as seen in the control animals. Several small-sized multilocular cells were observed between the unilocular adipocytes and/or perivascular spaces in the tissue (Fig 1D). These cells were polygonal in shape and had fine cytoplasmic vacuoles and round nuclei. Based on the appearance of the nuclei and immunohistochemical results for macrophages (described later), these isolated cells were
considered to be premature multilocular adipocytes. In addition, there were a number of characteristic structures (so called “crown-like structures”) that are composed of several foamy cells surrounding single unilocular adipocytes (Fig 1E). Each cell had a small, round to slightly elongated nucleus and there was no proliferating activity in any cells as confirmed with immunohistochemistry for Ki-67 (Fig 1F), as well as the negative reaction of multilocular adipocytes.

To confirm the contribution of macrophage infiltration to the crown-like structures, immunohistochemistry for CD68 was performed on adipose tissue from both Wistar lean and Wistar fatty rats. Almost no macrophages were found in the adipose tissue of Wistar lean rats (Fig 2A). On the other hand, CD68-positive macrophages were distributed throughout the adipose tissue of Wistar fatty rats (Fig. 2B). Some of these macrophages were scattered between unilocular adipocytes individually without any formation of clusters. Meanwhile, the majority of foamy cells forming crown-like structures were positive for CD68, although several CD68-negative cells, which were considered to be premature adipocytes, were also mixed in the structure (Fig. 2C). The immunohistochemistry confirmed that these surrounding cells were also positive for CD11b, a marker of granulocytes as well as macrophages (Fig. 2D). There were no remarkable differences from the control Wistar lean rats in other structures including vessels and thin connective tissues.
The 3-D observations of the adipose tissue components

Structural relationship of adipocytes, vasculature, and others

The 3-D observation of adipose tissue slices immunohistochemically stained for vimentin and fibronectin using fluorescent dyes allowed the spatial configuration of the adipocytes, the capillary network, and the interstitial mesenchymal cells to be seen.

In the Wistar lean rats, the capillary network spread over the surface of the unilocular adipocytes (Fig. 3A). The 3-D observations also confirmed the presence of vimentin-positive mesenchymal cells on the surface or between of the adipocytes, and these cells were encapsulated in fibronectin-positive extracellular matrix (ECM) on the surface of adipocytes (Fig. 3C). In the Wistar fatty rats, numerous multilocular adipocytes containing small lipid droplets were found on the surface of large unilocular adipocytes or beside the vasculature (Fig. 3B). These cells were found within the bundles of fibronectin (Fig. 3D) and were considered to be originating from vimentin-positive mesenchymal cells.
Discussion

The purpose of this experiment was to compare the histomorphological results of the adipose tissue from Wistar fatty rats with the findings reported for human obese subjects to explore the usefulness of this strain of rat as a disease model.

Within the adipose tissue of Wistar fatty rats, each unilocular adipocyte was hypertrophied, and several small-sized, multilocular adipocytes were observed between the unilocular adipocytes and/or perivascular spaces in the tissue. Hypertrophy of each adipocyte and hyperplasia of adipocytes are crucial phenomenon for the progression of obesity (Berry et al., 2014; Hausman et al., 2001), and these findings resembled the pathological changes in obese human subjects, as reported by others (Lijnen, 2011). Moreover, a characteristic structure consisting of several macrophages and surrounding a single unilocular adipocyte was observed frequently in the adipose tissue of Wistar fatty rats. This structure is called a crown-like structure (Apovian et al., 2008; Revelo et al., 2014), and it is well-known as a histological hallmark of the adipose tissue in obesity. Macrophages composing the crown-like structure are thought to infiltrate in order to remove the contents of dead adipocytes including triglycerides, and then, macrophages begin to show a foamy cytoplasmic appearance (Prieur et al., 2011). All of these results showed similar histopathology in the adipose tissue of obese human subjects and Wistar fatty rats. In addition, multilocular adipocytes appeared between the unilocular
adipocytes within the adipose tissue slices following the adipogenic stimulation in the organotypic culture system as shown in Chapter 1. Therefore, morphological changes during the adipogenesis in the author’s \textit{in vitro} model have been confirmed to be relevant to \textit{in vivo} obese animal model and human obese subjects.

Adipose tissue macrophages regulate the homeostasis of adipose tissue physiology via interfering with the adipocyte function by secreting cytokines (Kohlgruber and Lynch, 2015; Zeyda and Stulnig, 2007). In a healthy state, these macrophages polarize to the anti-inflammatory phenotype (M2 macrophage) and play roles in enhanced lipid handling and mitochondrial function or defense against a parasitic infection (Noel et al., 2004; Vieira-Potter, 2014). However, several researchers over the last decade revealed that adipose tissue macrophages working as the M2 type can switch to a pro-inflammatory phenotype (M1 macrophage) and this phenomenon contributes greatly on the onset and progression of obesity and obesity-related diseases (Apostolopoulos et al., 2015; Revelo et al., 2014); therefore, the obesity is now considered to be a kind of inflammatory disease. In this study, cells infiltrating the adipose tissue of Wistar fatty rats were considered to be mostly macrophages, based on the morphological observation and comparison with CD68- and CD11b-positive cell distribution. In obese subjects, neutrophils and lymphocytes, not only macrophages, form the inflammatory lesions in the adipose tissue, and all of these inflammatory cells may contribute to the
progression of the disease (Revelo et al., 2014). The details of the inflammatory response in the adipose tissue of Wistar fatty rats might be different from those in obese humans.

In conclusion, detailed observation of the adipose tissue of Wistar fatty rats utilizing immunohistochemistry and confocal laser microscopy revealed histomorphological homologies with adipose tissue of obese human subjects, and it was confirmed that the Wistar fatty rat is a useful model for studies on the obesity and obesity-related diseases.
Summary

The obesity is one of the most serious disorders in Western countries. Extensive studies have been conducted on the adipose tissue in obesity; however, the pathophysiology during disease progression is not fully understood. In this study, the detailed histomorphological examination of the adipose tissue of Wistar fatty rats, an obese/diabetic model, was performed using immunohistochemistry.

Within the adipose tissue of Wistar fatty rats, hypertrophy of each unilocular adipocyte was remarkable compared to the control Wistar lean rats, and several small-sized multilocular adipocytes were observed between the unilocular adipocytes and/or perivascular spaces in the tissue. In addition, a characteristic structure where several macrophages are surrounding single unilocular adipocytes (so-called “crown-like structure”) was observed frequently in the adipose tissue of Wistar fatty rats. Hypertrophy of each adipocyte and hyperplasia of adipocytes are crucial phenomenon as for the progression of obesity and the crown-like structure is well known as a histological hallmark of the adipose tissue in obesity. All of these results showed similarities of histopathology in the adipose tissue between the obese-human subjects and Wistar fatty rats.

Based on the above, histopathological characters of adipose tissue in Wistar fatty rats were
confirmed to be consistent with those for human obese subjects and it was considered that the

Wistar fatty rat is a useful animal model for studies on the obesity.
**Figure Legends**

Fig. 1. Histological differences of adipose tissue between Wistar lean rats and Wistar fatty rats. H-E-staining (A-E) and immunohistochemistry for Ki-67 (F, brown dye). Adipose tissue of Wistar lean rats was composed of mature adipocytes with a large lipid vacuole and a small flattened nucleus uniformly (A and B). In the Wistar fatty rats, hypertrophy of each unilocular adipocyte was remarkable and several small-sized multilocular adipocytes were observed between the unilocular adipocytes and/or perivascular spaces (C and D). In addition, there were a number of crown-like structures where several foamy cells are surrounding single unilocular adipocytes in Wistar fatty rats (E). There was no proliferating activity in any cells as confirmed with immunohistochemistry for Ki-67 (F). Scale bars = 10 μm for A and C, 50-μm for others.

Fig. 2. Immunohistochemistry for CD68 (A-C) and CD11b (D). Almost no macrophages were found in the adipose tissue of Wistar lean rats (A). CD68-positive macrophages were distributed through the adipose tissue of Wistar fatty rats (B). Several cells were scattered between unilocular adipocytes individually without any formation of clusters. On the other hand, the majority of foamy cells within crown-like structures were positive for CD68 (C). These macrophages were also positive with immunohistochemistry for CD11b (D). Scale bars = 50 μm.
Fig. 3. Immunohistochemistry of vimentin (A and B) and fibronectin (C and D). Several interstitial cells were found on the surface of unilocular adipocytes and within the bundle of fibronectin-positive matrixes in the Wistar lean rats (A and C). In the Wistar fatty rats, multilocular adipocytes containing small lipid droplets were found on the surface of large unilocular adipocytes or beside the vasculature within the bundles of fibronectin (B and D). Scale bars = 50 μm.
Fig. 1
Fig. 2
Section 2

Identification of Adipose Progenitor Cells and its Differentiation in

Adipose Tissue of Wistar Fatty Rats

Introduction

The obesity is a global health issue, and hypertrophy of each adipocyte and hyperplasia of adipocytes are crucial phenomenon during the progress of obesity (Berry et al., 2014). Although a large number of studies have been conducted for several decades, the precise localization of progenitor cells and the detail of process of adipose differentiation to mature adipocytes have not been fully cleared.

As mentioned in Chapter 1, using an in vitro organotypic culture system, the author has elucidated that the mesenchymal cells embedded in the extracellular matrix (ECM) around the mature adipocytes are a major source for adipogenesis in rat adipose tissue. In addition, histological 3-dimensional (3-D) observation using a confocal laser microscopy suggested the advent of CD105-expressing mesenchymal cells within the interstitium of the mature adipocytes under the adipogenic culture condition concurrent with the increment of adipocytes.
As a proof of concept regarding the phenomenon of adipogenesis which had been established in the \textit{in vitro} culture systems (Chapter 1), the author conducted detailed histomorphological examinations of the adipose tissue of Wistar fatty rats, an obese/diabetic model, using immunohistochemistry.
Materials and Methods

Animals

Forty-nine-week-old male Wistar fatty (obese/diabetic) and Wistar lean (control) rats were purchased from Takeda Rabics Ltd. (Kanagawa, Japan) and, under deep anesthesia with isoflurane (Escain, Mylan Inc., Tokyo, Japan), the animals were euthanized by exsanguination from the abdominal aorta/posterior vena cava. The care and use of these animals and the experimental protocols for this study were approved by the Animal Experimental Use Committee of Takeda Pharmaceutical Company Limited (where the author conducted this study).

Morphological evaluation of adipose tissue of Wistar fatty rats

The subcutaneous adipose tissue was removed from the inguinal region of the animals. To confirm the histomorphology, parts of the adipose tissues were fixed in 10 vol% neutral buffered formalin, embedded in paraffin, and sectioned at approximately 4-μm thickness. Sections were stained with hematoxylin-eosin (H-E) and examined under a light microscope. These results are described in section 1.

In addition, the double immunofluorescence staining was carried out on the adipose tissue of Wistar fatty and Wistar lean rats using anti-CD105 (anti-endoglin; abcam, Tokyo,
Japan) and anti-CD68 (anti-macrophage; Millipore, California, USA) antibodies. Briefly, sections were reacted with a mixture of these antibodies (CD105; 1:100, CD68; 1:1000) overnight at 4°C, and then reacted with each secondary antibody labeled with Alexa 488 and Alexa 594 (1:200; Molecular Probes, Oregon, USA) each for 30 min at room temperature, respectively. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; 1:000; Molecular Probes). These sections were observed using a confocal microscope system (LSM700; Carl Zeiss Japan, Tokyo, Japan).

Three-dimensional (3-D) observations using a confocal microscope

After removal from the animals, small pieces of adipose tissue were cut into 700-μm slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK). The adipose tissue slices were fixed overnight with 4 w/v% paraformaldehyde (4% PFA) at 4°C, rinsed in phosphate-buffered saline (PBS) and were pre-incubated with proteinase K (DAKO), methanol (Wako Pure Chemical Industries, Ltd.), and Tris buffered saline with Tween 20 (TBST; DAKO) sequentially. Samples were incubated with the following primary antibodies: anti-neuron-glial antigen 2 (NG2, 1:100, abcam), anti-Sox2 (1:100, abcam), or anti-CD105 (1:100) for 1 h at room temperature. After the incubation, and after the washing with TBST, the samples were reacted with a fluorochrome-conjugated secondary antibody (goat anti-rabbit Alexa Fluor 594 or goat anti-rabbit Alexa Fluor 488, 1:200; Molecular Probes) for 30
min at room temperature. Neutral lipids within the 4% PFA-fixed tissue slices were detected by a HCS Lipid TOX neutral lipid stain (Molecular Probes), and the endothelial cells and nuclei were stained with Alexa Fluor 594 conjugated GS-IB₄ (20 μg/mL; Molecular Probes) and DAPI, respectively. The 3-D structure and disposition of the adipose components were observed using a confocal microscope system (LSM700).
Results

The 3-D observations of the adipose tissue components

Immunohistochemistry for CD105, NG2, and Sox2; detection of mesenchymal stem cells

To evaluate the relationship of mesenchymal stem cells with the obesity, immunohistochemistry for CD105, NG2, and Sox2 was performed. In the Wistar lean rats, there were no positive reactions in any staining (Figs. 1A, 1C) except for the minor CD105-positive cells in the connective tissue (not shown). In the Wistar fatty rats, CD105-positive, non-endothelial cells, were observed beside the capillaries (Fig. 1B, arrows) or in the interstitium independent of the vasculature. In addition, several CD105-positive cells were involved at the area of cell aggregation (Fig. 1B, inset). There was a slight increase of NG2-positive cells in the adipose tissue of Wistar fatty rats (Fig. 1D, arrows). Sox2-positive cells were not detected in the adipose tissue of Wistar fatty rats in this experiment.

Double-immunohistochemistry for CD68 and CD105; correlation between macrophage aggregation and the presence of CD105-positive cells

The crown-like structure, which was observed in the adipose tissue of Wistar fatty rats under the light microscope, has been reported as a characteristic histological finding in human obesity subjects (Bremer et al., 2011). To confirm any correlation between the crown-like
structure and the distribution of CD105-positive cells, a double immunohistochemistry for CD68 and CD105 was conducted. In the adipose tissue of Wistar lean rats, there were almost no positive cells in the immunohistochemistry for CD68 or CD105 (Fig. 2A). In the Wistar fatty rats, there were several patterns indicating the presence of CD68- and CD105-positive cells throughout the adipose tissue; these included the distribution of CD105-positive cells on the surface of unilocular adipocytes without any correlation with CD68-positive macrophages (Fig. 2B) and crown-like structure formation only by CD68-positive cells (Fig. 2C) or by the mixture of CD68- and CD105-positive cells (Fig. 2D).
Discussion

As mentioned in Chapter 1, the author previously reported that adipose progenitor cells residing on mature adipocytes and the perivascular adipose progenitors were suggested to be sources for adipogenesis and the transition of the differentiation stage of the preexisting mesenchymal cells to stem state was revealed to be involved in adipogenesis in rat adipose tissue using *in vitro* organotypic culture. This experiment was a proof of concept study regarding the above phenomenon during the progression of obesity *in vivo* with detailed histomorphological examinations of the adipose tissue from Wistar fatty rats, an obese-diabetic model.

Small-sized multilocular adipocytes were observed between the unilocular adipocytes and/or perivascular spaces in the H-E-stained histology section of adipose tissue of Wistar fatty rats. These cells were also confirmed by the 3-D observation of tissue slices stained with several immunofluorescent stains. As discussed in Section 1, there was no proliferating activity in any cells as confirmed with immunohistochemistry for Ki-67, and multilocular adipocytes were considered to be derived from the preexisting cells. The progenitor cells of these multilocular adipocytes were vimentin-positive mesenchymal cells embedded in the fibronectin-positive ECM supporting mature adipocytes. These observations were confirmed in adipose tissue slices under the adipogenic stimulations in the previous *in vitro* experiment by the author (Chapter 1).
In that study, the CD105-positive, non-endothelial cells that were trans-differentiated/de-differentiated to the stem state from the preexisting state within the adipose tissue, are regarded as a source of adipose progenitor cells in the adipogenic tissue culture. To confirm the transference of experimental in vitro phenomenon to an in vivo obese situation, immunohistochemistry for CD105, NG2, and Sox2 were conducted on the adipose tissues of Wistar fatty rats. As a result, CD105- and NG2-positive mesenchymal cells were found on the surface of unilocular adipocytes in Wistar fatty rats. CD105 and NG2 are considered to be important markers of undifferentiated mesenchymal cells (De Schauwer et al., 2011; Russell et al., 2013) and CD105 expression would be related to the capacity for adipose differentiation (Zych et al., 2014). Furthermore, the presence of adipose stem cells is thought to have a role in adipogenesis (Cawthorn et al., 2012; Kim et al., 2014). Overall results from in vitro (Chapter 1) and in vivo (Chapter 2) experiments by the author show that the mesenchymal cells at stem state are the source of preadipocytes, and that this cascade might be involved in adipogenesis in the obesity.

The factors affecting differentiation of mesenchymal cells are unclear. One possibility is hypoxia. Several reports present the possibility that the hypoxia may contribute to the dysregulation of adipose tissue homeostasis in the obesity, because of the severe hypertrophy of adipocytes (Guglielmi et al., 2015; Revelo et al., 2014); the hypoxia condition may induce the
proliferation or regeneration of mesenchymal stem cells (Kim et al., 2014; Palumbo et al., 2014).

Another possibility is enhancement of stemness by infiltrating macrophages via the modulation of components of ECM. CD68-positive macrophages were observed in the adipose tissue of Wistar fatty rats diffusely. The ECM is largely modulated by infiltrating macrophages (Catalan et al., 2012), and in turn, the ECM regulates the proliferation activity of mesenchymal cells as well as cell differentiation (Gattazzo et al., 2014). There is a possible relationship between macrophage infiltration and CD105-/NG2-positive cells; however, the crown-like structure itself was not judged to correlate with the increment of mesenchymal cells at stem state based on the observations of double immunohistochemistry for CD68 and CD105.

In conclusion, the detailed observation of the adipose tissue of Wistar fatty rats utilizing the immunohistochemistry and confocal laser microscopy revealed the characteristic findings of adipose tissue related to the obese. The results of this study showed that the mesenchymal cells at stem state within the adipose tissue might be the source of preadipocytes, as previously confirmed in vitro (Chapter 1), and this cascade is involved in adipogenesis in obesity.
Summary

The obesity is a global health issue, and the hypertrophy of each adipocyte and hyperplasia of adipocytes are crucial phenomenon during the progress of obesity. Despite a large number of studies have been conducted for several decades, the precise localization of progenitor cells and the detail of process of adipose differentiation to mature adipocytes have not been fully cleared. In this study, the author conducted detailed histomorphological examination on the adipose tissue of Wistar fatty rats, an obese/diabetic model, using the immunohistochemistry.

All the results showed similarities of histopathology in the adipose tissue between obese human subjects and Wistar fatty rats. Immunohistochemical staining confirmed that the mesenchymal cells embedded in the ECM around the mature adipocytes were a major source for adipogenesis, and concurrently CD105- and NG2-positive mesenchymal cells were found on the surface of unilocular adipocytes in Wistar fatty rats. Collectively, the present detailed observation of the adipose tissue of Wistar fatty rats revealed characteristic findings of adipose tissue related to the obese. These results showed that the mesenchymal cells at stem state within the adipose tissue might be the source of preadipocytes, as previously confirmed in vitro, and this cascade is involved in adipogenesis in the obesity.
Figure Legends

Fig. 1. Immunohistochemistry of CD105 (A and B) and NG2 (C and D), both cell surface markers of mesenchymal stem cells. In the Wistar lean rats, there was no positive reaction in any staining (A and C). In the Wistar fatty rats, CD105-positive, non-endothelial cells were observed beside the capillary (B, arrows) or in the interstitium independent of the vasculature. In addition, several CD105-positive cells were involved at the area of cell aggregation (B, inset). There was a slight increase of NG2-positive cells in the adipose tissue of Wistar fatty rats (D, arrows). Scale bars = 50 μm.

Fig. 2. Double immunohistochemistry of CD68 and CD105. There were no positive cells in the adipose tissue of Wistar lean rats (A). In the Wistar fatty rats, there were several patterns including the distribution of CD105-positive cells on the surface of unilocular adipocytes without any correlation with CD68-positive macrophages (B) and crown-like structure formation only by CD68-positive cells (C) or by the mixture of CD68- and CD105-positive cells (D). Scale bars = 50 μm
Fig. 1
Fig. 2
Chapter 3

Effects of Lithium Chloride, a GSK-3β Inhibitor, on the Adipose Differentiation from Preadipocytes
Introduction

The obesity plays a key role in the etiology of metabolic diseases which are associated with type 2 diabetes, hypertension, and atherosclerosis (Nie and Sage, 2009). Medications including orlistat (a pancreatic lipase inhibitor) and lorcaserin (serotonin-2c receptor agonist) have been approved for the treatment of obesity; however, the classes of anti-obesity drugs are still limited. Therefore, the studies on the development of novel drugs are important.

For drug discovery, appropriate research tools to assess the pharmacological effects of the candidate compounds are required. The cell lines of preadipocytes such as 3T3-L1 and 3T3-F442A have been used in in vitro experiments to confirm the anti-adipogenic effects of molecules. Meanwhile, recent studies revealed that the adipose tissue contains a large number of multipotent stem cells (Baer and Geiger, 2012; Zuk et al., 2002), and the adipose-derived stem cells (ASCs) have been considered to be a major source for adipogenesis. In addition, the extracellular matrix (ECM) has important functions for maintaining the cell morphology and cell fate of the adipocytes (Gattazzo et al., 2014; Huang and Greenspan, 2012; Mariman and Wang, 2010), and therefore, there might be a gap in the differentiation state of preadipocytes between in vivo samples and in vitro experiments using isolated cell lines.

As mentioned in Chapter 1, the organotypic culture has the advantage of maintaining
physiological interactions between the cells and also structural integrity. To fill the potential gap in the adipogenesis between in vivo samples and classical in vitro experiments, the author has established the simple organotypic culture method of adipose tissue slices of rats as described in Chapter 1. In this method, the adipose tissue slices are prepared using a tissue chopper and these slices are cultured at the interface between the air and a culture medium, and the adipose differentiation of the mesenchymal cells between or on the surface of mature adipocytes induced by a cocktail including insulin, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX) has been confirmed. Further, as shown in Chapter 2, morphological changes and the transition of the cell differentiation state observed during the adipogenesis in this in vitro model have been confirmed to be relevant to an in vivo obese animal model.

To investigate the usefulness of an organotypic culture system using adipose tissue slices as an in vitro model for compound screening of anti-adipogenic effects, lithium chloride, which is a glycogen synthase kinase (GSK)-3β inhibitor and has been reported as an anti-adipogenic agent in vitro and in vivo (Aratani et al., 1987; Lee S. et al., 2013; Naito et al., 2012; Zaragosi et al., 2008), was added to the culture medium before adipogenic stimulation and the histomorphological evaluation of adipose tissue slices was conducted.
Materials and Methods

Organotypic culture of adipose tissue slices

The procedures for the organotypic culture of adipose tissue slices were established by the author as shown in Chapter 1.

Six-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and, under deep anesthesia with isoflurane (Escain, Mylan Inc., Tokyo, Japan), the animals were euthanized by exsanguination from the abdominal aorta. The care and use of these animals and the experimental protocols for this study were approved by the Animal Experimental Use Committee of Takeda Pharmaceutical Company Limited (where the author conducted this study). The subcutaneous adipose tissue was removed from the inguinal region of the animals. Small pieces of adipose tissue were rinsed with DMEM-F12 medium (Gibco, Life Technologies Japan, Ltd., Tokyo, Japan), and then cut into 700-μm slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK). The tissue slices (2 to 4 slices/well) were put onto a membrane insert (Millicell-CM; Nihon Millipore K.K., Tokyo, Japan) and placed in a 6-well culture dish containing 1 mL of DMEM-F12 medium supplemented with 10 vol% newborn calf serum (Gibco, Life Technologies Japan, Ltd.) (10% NCS/DMEM). The slices were incubated at 37°C
in a 5% CO₂ incubator for 2 days.

Adipogenic culture of adipose tissue slices and effects of lithium chloride on the adipogenesis

As a model of adipogenesis in vitro, the adipose tissue slices were incubated with 10% NCS/DMEM supplemented with 5 μg/mL insulin (Sigma-Aldrich Japan K.K., Tokyo, Japan), 0.01 μg/mL DEX (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 2.2 μg/mL IBMX (Wako Pure Chemical Industries, Ltd.) for 2 days. This mixture has been reported to be a well-defined adipogenic cocktail in the studies using the 3T3-L1 preadipocyte cell line (Ntambi and Young-Cheul, 2000).

Lithium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the medium, in the above adipogenic condition, at the concentrations of 1 and 30 mM to examine its effects on the adipogenesis in vitro. The concentration levels were selected based on the information of the in vitro experiments showing anti-adipogenic effect of lithium chloride (Aratani et al., 1987; Zaragosi et al., 2008).

Morphological observation of adipose tissue slices

To confirm the histomorphology of the tissue slices, the adipose tissue slices before culture (Day 0) and on culture Day 2 were fixed in 10 vol% neutral buffered formalin and
embedded in paraffin. The whole slices were sectioned perpendicularly. Sections were stained with hematoxylin-eosin (H-E), and then examined under a light microscope.

**Three-dimensional (3-D) observations using a confocal microscope**

*Fluorescent vital staining*

On culture Days 0 and 2, triple staining using lipid, endothelial cells, and nucleic acid-specific fluorescent dyes was performed on unfixed adipose tissues according to the previously described procedure (Nishimura et al., 2007; Suga et al., 2009). Briefly, the tissue slices were incubated with the following reagents for 30 min at 37°C: BODIPY 558/568 (5 μg/mL) for lipid staining of the adipocytes, Alexa Fluor 488-conjugated *Griffonia simplicifolia* isolectin (GS-IB₄, 20 μg/mL) for endothelial cells, and Hoechst 33342 (10 μg/mL) for the nuclei. All of these reagents were purchased from Molecular Probes (Eugene, Oregon, USA).

*Whole-mount immunohistochemistry*

Whole-mount immunohistochemistry was performed on culture Days 0 and 2. The adipose tissue slices were fixed overnight with 4 w/v% paraformaldehyde (4% PFA) at 4°C and rinsed in phosphate-buffered saline (PBS). The tissue slices were pre-incubated with proteinase K (DAKO), methanol (Wako Pure Chemical Industries, Ltd.), and Tris buffered saline with Tween 20 (TBST; DAKO) sequentially. Samples were incubated with the following primary
antibodies: anti-CCAAT-enhancer-binding protein-β (C/EBP-β; 1:50; Bioworld Technology, Inc.,
Minnesota, USA), anti-fibronectin (1:100; DAKO), or anti-CD105 (anti-endoglin; 1:50; abcam)
for 1 h at room temperature. After the incubation, and after washing with TBST, the samples
were reacted with a fluorochrome-conjugated secondary antibody (goat anti-rabbit Alexa Fluor
594 or goat anti-rabbit Alexa Fluor 488, 1:200; Molecular Probes) for 30 min at room
temperature. Neutral lipids within the 4% PFA-fixed tissue slices were detected by a HCS Lipid
TOX neutral lipid stain (Molecular Probes), and the endothelial cells and nuclei were stained
with Alexa Fluor 594 conjugated GS-IB4 (20 μg/mL; Molecular Probes) and
4,6-diamidino-2-phenylindole (DAPI; 1:000; Molecular Probes), respectively.

**Confocal microscope**

The 3-D structure and disposition of the adipose components were observed using a
confocal microscope system (LSM700; Carl Zeiss Japan, Tokyo, Japan). The multistep
observations were performed from the cut surface of the core portion of the tissue slices.
Results

Histological features after the organotypic culture of adipose tissue slices with adipogenic stimulation and the inhibition of adipogenesis by lithium chloride

Adipose tissue slices after the 2-day culture with basic medium were composed of numerous mature adipocytes containing large unilocular fat droplets with elongated nuclei and vessels including capillaries and small-sized arteries/veins (Fig. 1A). As shown in Chapter 1, after the adipogenic stimulation with a mixture of insulin, DEX, and IBMX, small-sized multilocular adipocytes appeared between the unilocular-mature adipocytes and/or perivascular spaces in the tissue slices on Day 2 (Fig. 1B). Those newly formed multilocular cells were polygonal to spindle-shaped and had fine cytoplasmic vacuoles and round nuclei, indicating the adipogenic differentiation of the mesenchymal cells residing on the mature adipocytes (Chapter 1).

Supplementation of lithium chloride at 1 mM did not show any effect on the histomorphology of adipose tissue slices with adipogenic stimulation (Fig. 1C). With supplementation of lithium chloride at 30 mM, multilocular adipocytes having small cytoplasm containing fine lipid droplets were occasionally observed between unilocular mature adipocytes; however, the cytoplasmic area of these cells was smaller than those for multilocular cells found
within the adipose tissue slices in the other condition (adipogenic stimulation only or adipogenic stimulation with lithium chloride at 1 mM) and the number of multilocular adipocytes were remarkably decreased in the tissue slices subjected to the supplementation of lithium chloride at 30 mM (Fig. 1D).

**The 3-D observations of the adipose tissue components**

*Effects of adipogenic stimulation on the morphology of adipose tissue slices and inhibitory effects of lithium chloride on adipogenesis*

The 3-D observation of whole adipose tissue slices stained with 3 fluorescent dyes visualized the spatial configuration of the adipocytes and the capillary network (Pre-culture, Fig. 2A). Confocal microscopy confirmed that there were no obvious changes in the adipose tissue components in the tissue slices after the 2-day culture with the basic medium (Fig. 2B).

The observations of the tissue slices after adipogenic stimulation revealed developing fine lipid droplets in the interstitial cells in the perivascular regions or on the surface of the mature adipocytes, and there are several cells with large lipid droplets that were considered to be the product of the fine droplets (Fig. 2C).

Although supplementation with lithium chloride at 1 mM did not show any effect on the morphology within the adipose tissue slices exposed to adipogenic stimulation, lithium
chloride at 30 mM showed clear inhibiting effects on the adipogenesis induced by the cocktail of insulin, DEX, and IBMX (Fig. 2D). Several interstitial cells containing fine lipid droplets within their cytoplasm were present between or on the surface of large unilocular adipocytes and there was not a complete inhibition of adipogenesis; however, the size of each lipid droplet was much smaller compared to those found in the interstitial cells within the tissue slices exposed to the adipogenic culture without lithium chloride.

**Evaluation of adipose differentiation of interstitial cells: immunohistochemistry for C/EBP-β**

For the characterization of adipogenetic transformation in the interstitial cells, expression of C/EBP-β, which is known to be induced in the early stage of adipocyte differentiation (Farmer, 2005; Hung et al., 2004; Lee K. et al., 1998), was investigated.

C/EBP-β-positive cells were abundantly increased between or on the surface of large unilocular adipocytes after the 2-day adipogenic culture along with an increase in the number of multilocular adipocytes, and in the tissue slices cultured with adipogenic stimulation and supplementation with lithium chloride at 30 mM (Figs. 3A-D).

**Immunohistochemistry for CD105: detection of mesenchymal stem cells or cells at stem state**

As described in Chapter 1, a limited number of CD105-positive cells were found in the adipose tissue slices on pre-culture (Fig. 4A) and after the 2-day culture with basic medium (Fig.
After the 2-day adipogenic culture, CD105-positive, non-endothelial cells appeared in the tissue slices (Fig. 4C). Regarding the detailed localization of CD105-positive cells, some of the positive cells were present very near the capillary network, but many of the cells were present in the interstitium independent of the vasculature. These cells appeared similarly in the adipose tissue slices cultured with adipogenic stimulation and supplementation with lithium chloride at 30 mM (Fig. 4D) and there were no clear effects of lithium chloride on their appearance.
Discussion

During the discovery phase of pharmaceutical development, several \textit{in vitro} models are used to assess the pharmacology, drug metabolism, or toxicity of the drug candidates in development. These models have several useful points such as the high-throughput or the prediction of \textit{in vivo} human events; however, the author confronted the gaps between \textit{in vitro} and \textit{in vivo} results, and a study to bridge these gaps has been conducted (Joris et al., 2013; Vellonen et al., 2014). In the study on adipogenesis, preadipocyte cell lines such as 3T3-L1 and 3T3-F442A or the stromal vascular fraction (SVF), which is a heterogeneous cell mixture isolated by the enzymatic dissociation of adipose tissue, have been used in \textit{in vitro} experiments (Ntambi and Young-Cheul, 2000; Sengenes et al., 2005). However, it has been reported that ECMs have important functions for maintaining the cell morphology and cell fate of the adipocytes (Gattazzo et al., 2014; Huang and Greenspan, 2012; Mariman and Wang, 2010), and there might be a gap in the biology of adipogenesis including the differentiation state of the mesenchymal cells between \textit{in vivo} samples and \textit{in vitro} experiments using isolated cells (Zeve et al., 2009).

To bridge these gaps, the author has developed the organotypic culture method of adipose tissue slices as described in Chapter 1. In this culture using rat adipose tissue, the adipose differentiation of the mesenchymal cells between or on the surface of mature adipocytes
was confirmed under adipogenic stimulation. Also, as shown in Chapter 2, morphological changes and the transition of cell differentiation state observed during the adipogenesis in this *in vitro* model have been confirmed to be relevant to an *in vivo* obese animal model. For further investigation of the author’s culture method as a research tool for the assessment of adipogenic or anti-adipogenic compounds, lithium chloride, which is a GSK-3β inhibitor and has been reported as an anti-adipogenic agent *in vitro* and *in vivo* (Aratani et al., 1987; Lee S. et al., 2013; Naito et al., 2012; Zaragosi et al., 2008), was added to the culture medium concomitant with adipogenic stimulation and the histomorphological evaluation of adipose tissue slices was conducted.

In the histopathological evaluation of H-E-stained adipose tissue slices, small-sized multilocular adipocytes appeared between the unilocular-mature adipocytes and/or perivascular spaces following the adipogenic stimulation with a mixture of insulin, DEX, and IBMX. With supplementation of lithium chloride at 30 mM, the cytoplasmic area of these cells was smaller than those for multilocular cells found within the adipose tissue slices in the other condition (adipogenic stimulation only or adipogenic stimulation with lithium chloride at 1 mM) and the number of multilocular adipocytes was remarkably decreased. In the 3-D observation of adipose tissue slices using confocal microscopy, adipogenic stimulation induced development of fine to large lipid droplets in the interstitial cells in the perivascular regions or on the surface of the
mature adipocytes, and lithium chloride at 30 mM showed clear inhibiting effects on the adipogenesis induced by adipogenic stimulation. Based on these observations, it was confirmed that the high concentration of lithium chloride has anti-adipogenic effects as reported previously (Aratani et al., 1987; Zaragosi et al., 2008), and further, the organotypic culture method developed by the author was also confirmed to be a useful in vitro research tool for the discovery of anti-obesity pharmaceuticals.

In this experiment, the inhibition of adipogenesis by lithium chloride was not complete and several interstitial cells containing fine lipid droplets were observed between or on the surface of large unilocular adipocytes even after the lithium chloride treatment at 30 mM. The transition of gene expression during adipogenesis has been well-investigated using an in vitro system of 3T3-L1 preadipocytes (Ntambi and Young-Cheul, 2000) and a transcription factor, C/EBP-β is known to be induced in the early stage of adipocyte differentiation (Farmer, 2005; Hung et al., 2004; Lee K. et al., 1998). In addition, as shown in Chapter 1, the CD105-positive cells that were trans-differentiated/de-differentiated to the stem state from the preexisting state within the adipose tissue appeared at the interstitium during adipogenesis. Newly formed adipocytes within adipose slices were positive for C/EBP-β- and CD105-positive cells were observed at the interstitium; therefore, exposure to lithium chloride did not inhibit the early phase of adipose differentiation from the mesenchymal cells but exposure to lithium chloride
did inhibit adipogenesis during the late phase differentiation of adipocytes under the conditions of this experiment. At this point, it is unclear whether the compound concentration was insufficient to inhibit adipogenesis completely or lithium chloride can only inhibit the late phase of adipogenesis.

Lithium chloride was used in this experiment as a GSK-3β inhibitor. Evidence supports the theory that the Wnt/β-catenin pathway is an important regulator of adipocyte differentiation (Li et al., 2008), and this pathway affects the cellular functions by regulating β-catenin levels and its subcellular localization. Although cytoplasmic β-catenin protein is constantly degraded by GSK-3β in the absence of the Wnt ligand (Naito et al., 2012), binding of the Wnt ligand to the receptor induces the β-catenin accumulation in the cytoplasm and subsequent translocation to the nucleus, resulting in the activation of the Wnt target genes. The treatment with DEX causes inhibition of Wnt/β-catenin signaling, activation of GSK-3β, and finally downregulation of β-catenin protein (Naito et al., 2012), and this effect is considered to be a critical event during the adipose differentiation of preadipocytes/mesenchymal cells. In addition to lithium chloride, a small molecule showed anti-adipogenic effects in \textit{in vitro} and \textit{in vivo} models via activation of Wnt/β-catenin signaling (Choi et al., 2013). The Wnt/β-catenin pathway has important roles in tissue homeostasis, cell proliferation, and cell differentiation in the whole body. Although it seems to be difficult to control the risk-benefit balance in
Wnt/β-catenin treatment, further experiments are needed in order to discover good anti-obesity drugs.

In conclusion, the anti-adipogenic effects of lithium chloride, a GSK-3β inhibitor, were confirmed as reported and an organotypic culture system using adipose tissue slices was confirmed to be a useful as an *in vitro* research tool for the investigation of adipose tissue biology and for the discovery of anti-obesity drugs.
Summary

For drug discovery, appropriate research tools to assess the pharmacological effects of the candidate compounds are required. The author has established an organotypic culture method using adipose tissue slices, and, for further investigation of this culture system as a research tool for the assessment of anti-adipogenic compounds, lithium chloride, which is a GSK-3β inhibitor, was added to the culture medium before adipogenic stimulation and the histomorphological evaluation of adipose tissue slices was conducted.

In the histopathological evaluation of H-E-stained adipose tissue slices, small-sized multilocular adipocytes appeared between the unilocular-mature adipocytes and/or perivascular spaces following the adipogenic stimulation. Supplementation with lithium chloride at 1 mM did not modify the effects of adipogenic stimulation; however, with supplementation of lithium chloride at 30 mM, the cytoplasmic area of newly formed multilocular cells were smaller than those for cells found in the other condition and the number of multilocular adipocytes was remarkably decreased. The 3-D observation of adipose tissue slices using confocal microscopy also confirmed that lithium chloride at 30 mM showed clear inhibiting effects on the adipogenesis induced by adipogenic stimulation.

Based on these observations, it was confirmed that the high concentration of lithium
chloride has anti-adipogenic effects, and further, the organotypic culture method developed by the author was also confirmed to be a useful *in vitro* research tool for the investigation of adipose tissue biology and for the discovery of anti-obesity pharmaceuticals.
**Figure Legends**

Fig. 1. Effects of adipogenic stimulation on the histomorphology of adipose tissue slices and its modulation by lithium chloride. H-E staining. Adipose tissue slices after the 2-day culture with basic medium were composed of mature adipocytes with a large lipid vacuole and a small flattened nucleus uniformly (A). Following the adipogenic stimulation, polygonal multilocular adipocytes appeared between mature adipocytes (B). Supplementation of lithium chloride at 1 mM did not show any effects on the histomorphology of adipose tissue slices with adipogenic stimulation (C); however, the number of multilocular adipocytes was remarkably decreased in the tissue slices with supplementation of lithium chloride at 30 mM (D). Scale bars = 25 μm.

Fig. 2. Fluorescent staining of whole adipose tissue slices. Before culture, the close connection between each adipocyte (red) and the capillary network (green) was confirmed (A), and there were no obvious differences in morphology of adipose tissue slices after the 2-day culture with basic medium (B). After the 2-day adipogenic culture, numerous multilocular cells having very small lipid droplets appeared between or on the surface of the mature adipocytes or beside the vasculature (C). Supplementation with lithium chloride at 30 mM showed clear inhibiting effects on the adipogenesis induced by the adipogenic stimulation (D). Scale bars = 50 μm.
Fig. 3. Immunohistochemistry for C/EBP-β. Along with the increase in the number of multilocular adipocytes, C/EBP-β-positive cells (indicated by red fluorescence in the nucleus) were increased with adipogenic culture and not affected by the supplementation of lithium chloride. Scale bars = 50 μm.

Fig. 4. Immunohistochemistry for CD105, a cell surface marker of mesenchymal stem cells. In the pre-culture slices (A) and after the 2-day culture with basic medium (B), the positive reaction was not remarkable. On Day 2 of the adipogenic culture (C), CD105 expression was up-regulated in the several cells beside the capillary network or interstitial cells that were present independently from the vasculature. These CD105-positive cells were also observed in the adipogenic-cultured slices with lithium chloride supplementation (D). Scale bars = 50 μm.
Fig. 2
Fig. 3
Fig. 4
Conclusions

To explore the origin of adipocytes (preadipocytes) and underlying mechanism of adipogenesis, a series of in vitro and in vivo studies were conducted. Based on the results and the findings, the following conclusions are drawn:

1. The author successfully established the organotypic culture system of the adipose tissue from rats for an appropriate investigation system in vitro, and this culture system was confirmed to be a useful tool for adipose tissue research.

2. The results of in vitro evaluation using an organotypic culture system of adipose tissue slices showed that the adipose progenitor cells residing on mature adipocytes as well as the perivascular adipose progenitors were suggested to be a source for adipogenesis, and that the transition of the differentiation stage of the preexisting mesenchymal cells to stem state was revealed to be involved in adipogenesis in rat adipose tissue.

3. Detailed observation of the adipose tissue of Wistar fatty rats utilizing immunohistochemistry and confocal laser microscopy revealed histomorphological homologies with adipose tissue of obese human subjects, and it was confirmed that the Wistar fatty rat is a useful model for studies on the obesity and obesity-related diseases.
4. Histomorphological evaluation of adipose tissue of Wistar fatty rats showed that the mesenchymal cells at stem state within the adipose tissue are the source of preadipocytes, as previously confirmed \textit{in vitro}, and this cascade is involved in adipogenesis in obesity.

5. The high concentration of lithium chloride, a GSK-3\(\beta\) inhibitor, showed anti-adipogenic effects as reported, and the organotypic culture method developed by the author was also confirmed to be an useful \textit{in vitro} research tool for the investigation of adipose tissue biology and for the discovery of anti-obese pharmaceuticals.
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