Adiponectin is partially associated with exosomes in mouse serum

**(マウスの血清においてアディポネクチンの一部は
エキソソームに存在する)**

**A thesis submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
in the field of Applied Bioscience
Graduate School of Agriculture
Hokkaido University**

Worrawalan Phoonsawat

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**Special Postgraduate Program in
Biosphere Sustainability Science
Division of Applied Bioscience
Graduate School of Agriculture
Hokkaido University
Doctor Course 2014**

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ABSTRACT

Exosomes are membrane vesicles with a size of 30–120 nm that are released by many different cell types. They have been found in many biological fluids including blood, bronchoalveolar lavage fluid, urine, bile, and breast milk. Exosomes harbor a wide variety of proteins, lipids, mRNA, and microRNA, which can be transferred to another cell, and are implicated in cell-to-cell communication by transferring molecules. Thus, exosomes play an important role in physiological and pathophysiological processes and also are applicable as a source of disease biomarkers.

Previous studies suggest that adipocyte-derived exosomes play a role in cell-to-cell communication during the development of metabolic diseases. However, the characteristics and function of exosomes released from adipocytes *in vivo* remain to be elucidated. Clearly, exosomes released from adipocytes could exist in the circulation. In addition, because the composition of exosomes is heterogenic, depending on the cellular origin of the exosome, adipocyte-derived exosomes could be accompanied by molecules produced specifically in adipocytes. In this context, this study postulated that such molecules associated with exosomes in the serum could be markers for adipocyte-derived exosomes *in vivo*. This study particularly focused on secretory proteins produced specifically in adipocytes, namely adipocytokines including adiponectin, leptin, and resistin.

1. Serum adiponectin is partially associated with exosomes

Based on western blotting, CD63, a well-known protein marker of exosomes, was concentrated in the pellet of mouse serum after ultracentrifugation, suggesting successful isolation of exosomes. Western blotting detected adiponectin but no leptin and only trace amounts of resistin in the exosome fraction. After ultracentrifugation on a discontinuous gradient, both adiponectin and CD63 were detected in a fraction at a density of 1.17 g/mL, consistent with the density of exosomes. The adiponectin signal in the exosome fraction was decreased by proteinase K treatment and completely quenched by a combination of proteinase K and Triton X-100. These results suggest that a portion of adiponectin exists as a transmembrane protein in the exosomes in mouse serum.

2. Exosome-associated adiponectin may be a physiologically relevant form

Adiponectin exists as low-molecular-weight (LMW), middle-molecular-weight (MMW) and high-molecular-weight (HMW) forms in the circulation, and the latter has more relevant roles in its physiological functions (e.g., protecting metabolic diseases). Western blotting following SDS-PAGE under nonreducing and unheated conditions indicated that, although MMW forms are the predominant form in the serum, HMW forms are present principally in the exosome fraction. These findings suggest that exosome-associated adiponectin and exosome-free adiponectin may have different physiological and pathological functions *in vivo*. Indeed, quantitative ELISA showed that the concentration of adiponectin in the serum and the ratio of adiponectin to total protein in the exosome fraction were lower in obese mice than in lean mice.

In conclusion, this study showed that serum adiponectin is partially associated with exosomes in mice. Considering that adiponectin is produced exclusively by adipocytes, adiponectin-associated exosomes in serum could be derived from adipocytes. This study proposes that adiponectin could be a marker for exosomes released from adipocytes *in vivo*.

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LIST OF ABBREVIATIONS

AMPK	Adenosine monophosphate-activated protein kinase
BCA	bicinchoninic acid assay
CCL2	chemokine (C-C motif) ligand 2
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
C/EBPs	CCAAT-enhancer-binding proteins
DW	distilled water
ELISA	enzyme-linked immunosorbent assay
EMV	exosomes and microvesicles
EpCAM	epithelial cell adhesion molecule
ER	endoplasmic reticulum
Ero	ER oxidoreductase
ESCRT	endosomal sorting complexes required for transport
FIZZ	found in inflammatory zone
HMW	high-molecular-weight
HSPA8	heat shock protein 70 kDa protein 8
ICAM-1	intracellular adhesion molecule-1
IGF-1	insulin-like growth factor-1
IL-6	interleukin 6
IL-10	interleukin 10
IRS	insulin receptor substrate
JAK	Janus kinase
LMW	low-molecular-weight
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
miRNA	micro ribonucleic acid
MAPK	mitogen-activated protein kinase

MMW	middle-molecular-weight
MVB	multivesicular bodies
NF-κB	nuclear factor for the k immunoglobin light chain in B cells
PBS	phosphate-buffered saline
PI3K	phosphatidyl-inositol 3'-kinase
PPRP	peroxisome proliferator-activated receptor
RELM	resistin-like molecules
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SREBP1c	sterol regulatory element binding protein 1c
SIRT 1	sirtuin 1
SVF	stromal vascular fraction
STAT	signal transducer and activator of transcription
Th1 cells	type 1 helper T cell
TLR	toll-like receptor
TNF- α	tumor necrosis factor alpha
TZDs	thiazolidinediones

CHAPTER I
INTRODUCTION

1.1 Exosomes

1.1.1 Definition

Exosomes are usually defined as 30-100 to 200 nm diameter vesicles, released by many cell types (Müller, 2012; Silverman et al., 2010; Witwer et al., 2013). Similar to microvesicles, ectosomes, and apoptotic bodies, exosomes are present in biological fluids (e.g. blood, urine, ascites, saliva, semen, nasal secretion, bile, breast milk, and cerebrospinal fluid), cells (e.g. B-cells, T-cells, and dendritic cells), cell cultured-conditioned media, and tissues, such as adipose tissue and thymus. They also contain protein complexes, lipoprotein particles, cytokines, and RNA including microRNA (miRNA) and messenger RNA (mRNA). Apart from size, exosomes can be distinguished from other extracellular vesicles by source of origin. Exosomes originated from endosomes bud into the luminal spaces, resulting in the formation of multivesicular bodies (MVB) and released into either the extracellular space for direct communication between cells, or through blood circulation as shown in Fig. 1 (Bhatnagar et al., 2007; Müller, 2012; Silverman et al., 2010). Moreover, exosomes contain conserved as well as cell-type specific sets of exosomal proteins such as heat shock protein 70 kDa protein 8 (HSPA8) and tetraspanin CD63, proteins link to endosomal system (e.g. different Rab- GTPases or Tsg101 of the ESCRT I complex), to cytoskeleton (e.g. β-actin, moesin, cofillin and tubulins) and to the organization of membrane microdomains (e.g. the tetraspanins CD9, CD81 and CD82 or the flotillins Flot1 and Flot2) as well as antigen presenting cells and MHC class I and II as shown in Fig. 2 (Ludwig & Giebel, 2012).

1.1.2 Choice of samples

To date, many research studies have found the presence of exosomes in blood, body fluids (e.g. urine, saliva, and breast milk), and cell cultured conditioned

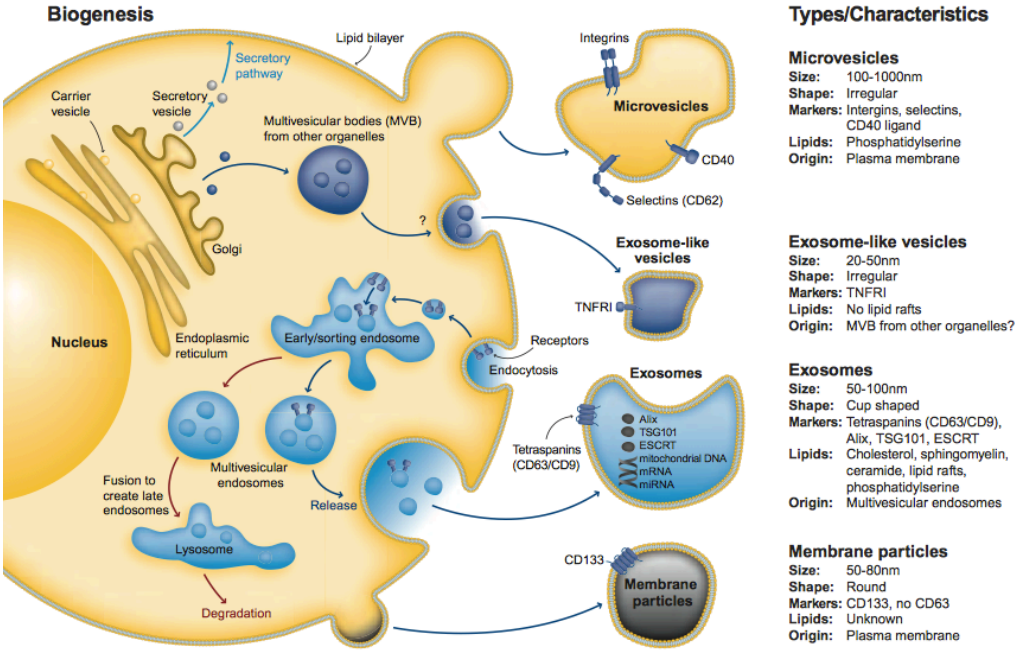


Fig. 1 : Biogenesis of exosomes and extracellular vesicles (http://docs.abcam.com/pdf/general/secreted_extracellular_vesicles_web.pdf)

a) b)

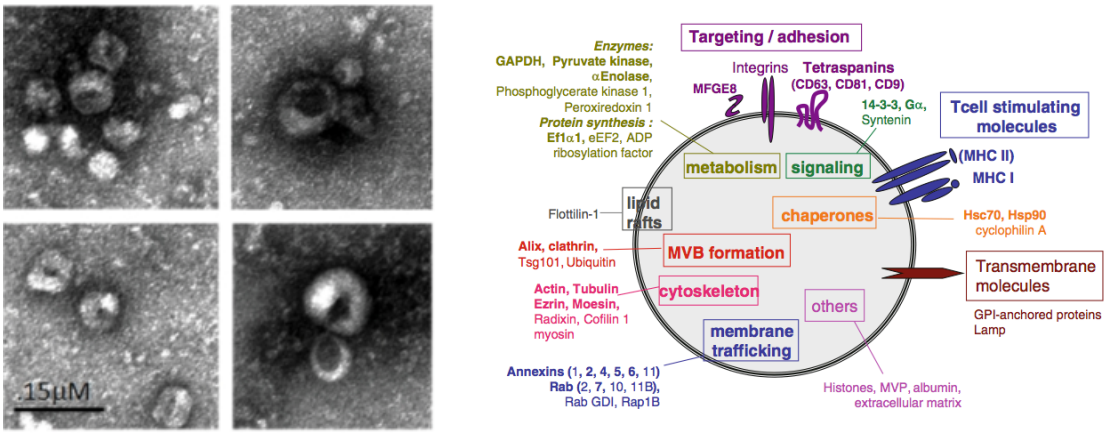


Fig. 2 : Characteristics of exosomes. (a) electron microscopy of the ultracentrifugation pellet from serum shows the characteristic spherical shape and size of exosomes (Gallo et al., 2012). (b) protein composition of a canonical exosomes (Chaput & Théry, 2010).

media as a potential diagnostic biomarkers for many diseases such as metabolic diseases, cardiovascular diseases, allergy-related diseases, and cancers. Peripheral blood is one of the effective samples in exosome study since exosomes may circulate and reflect the dysfunctional or disease stages of the relevant cells from which they originate. Concentrations of exosomes and microvesicles in peripheral blood of healthy probands are 5-50 μg/ml (Müller, 2012). Retrieved vesicles are consistently more abundant in sera than in plasma (about 3,000,000 exosomes per μl), and platelet-derived extracellular vesicles released after blood collection during the process of clot formation may account for over 50% of extracellular vesicles in serum (Vlassov et al., 2012; Witwer et al., 2013). Platelet-derived exosomes are enriched in CD63, a tetraspan protein also found on exosomes from other cell types (Denzer et al, 2000). The second largest population of blood exosomes and microvesicles (EMV) is derived from mononuclear phagocyte cell linage. A small percentage of blood EMV originates from T-cells, neutrophils, and endothelial cells (Müller, 2012). Apart from biological fluids which are represented *in vivo* study, *in vitro* study of exosomes released in conditioned media from many cell types helps us to understand further the physiological function of exosomes. Importantly, to avoid the contamination of exosomes from serum used in the cell culture media, the serum must be eliminated exosomes by ultracentrifugation at 100,000xg overnight before using in the experiment (Deng et al., 2009; Sun et al., 2010; Van Niel, 2006; Vlassov et al., 2012).

1.1.3 Isolation of exosomes

Exosomes can be isolated by many methods including differential centrifugation, filtration, immunoaffinity isolation, microfluidics techniques, and commercial kits. Choice of isolation method depends on how purity and concentration of exosomes are needed. The methods of exosome isolation are listed as follows.

1.1.3.1 Differential centrifugation

Differential centrifugation is the standard protocol for exosome isolation. Basically, for biofluids and conditioned media, dead cells and cell debris are removed at low speed centrifugation (300-2,000×g), and larger vesicles are removed at 2,000-20,000×g. Next, the sample solutions containing smaller vesicles are diluted with PBS

to reduce viscosity and subjected to ultracentrifugation at 100,000-200,000×g with fixed angle or swinging bucket rotor for about 2 times. However, the limitation of this method includes contamination from extravescular protein complexes, lipoprotein particles, and other contaminants (Momen-Heravi et al., 2013). Although the pellets are washed with PBS in order to remove these particles, it is difficult to completely remove them.

To further purify exosomes, sucrose gradient centrifugation is used to separate exosomes and contaminants based on its buoyant floatation density. Exosomes are separated in different concentrations of sucrose solution, sucrose cushion, or iodixanol (OptiPrep^{TM}) at the density range of 1.1-1.9 g/ml (Momen-Heravi et al., 2013; Tauro et al., 2012; Witwer et al., 2013).

1.1.3.2 Size exclusion

This method is used to isolate or concentrate exosomes based on particle size by passage through 0.2 or 0.8 μm filter. Size exclusion is often used to combine with other isolation methods. For example, samples are filtered before performing ultracentrifugation. Low protein binding-size exclusion filters such as hydrophilized polyvinylidene difluoride membrane are used to isolate and enrich exosomes. However, this method may damage large vesicles and platelet and possibly cause the skewing result (Momen-Heravi et al., 2013; Witwer et al., 2013).

1.1.3.3 Immunoaffinity isolation

Exosomes are isolated based on the presence of surface proteins markers enriched on exosomes. Antibodies to surface proteins are associated with magnetic beads or other media and used to select the desired exosomes by capturing with selective protein markers (e.g. CD63, A33, MHC class II, and EpCAM). However, this method is not recommended for functional studies since elution of exosomes from the antibody and media seems to be difficult. It is also not recommended for large amount of samples (Chaput & Théry, 2010; Momen-Heravi et al., 2013; Witwer et al., 2013).

1.1.3.4 Polymeric precipitation

Recently, commercial reagents became available to isolate enriched exosomes with shorter time than standard ultracentrifuge method. The principle is based on polymeric precipitation of biofluid with reagent for 30 min to overnight, followed by low speed centrifugation to collect supernatant, which contains exosomes. In a recent study, the highest yield of exosomes was achieved using ultracentrifugation with ExoQuick^{TM} precipitation, whereas higher quality exosomes isolation with intact morphological structures was achieved by ultracentrifugation with density gradient centrifugation (Momen-Heravi et al., 2013). Polymer-based precipitation may produce high yield of exosomal RNA and may be appropriate in the case of fluids that are thought to have enriched small extracellular vesicle fractions, but the rigorous assessment of contaminating particle fractions is recommended when this method is employed (Witwer et al., 2013)

1.1.3.5 Microfluidics

Microfluidics is the study and manipulation of fluid flow at the microscale. The principle of separation is based on frictional force, which offers the unique options for control of separation, reaction and measurement processes that are unavailable at the macroscale, as well as offering significant advantages by reduction material costs, energy consumption and sample sizes, while increasing throughput and permitting multiplexing for many familiar laboratory processes. Lab-on-chip microfluidic device is operated by specific binding of exosomes to antibody-coated surface. The biofluid of interest is then loaded on a pump that slowly pushes fluid through the chip, allowing targeting isolation of exosomes (Momen-Heravi et al., 2013).

1.1.4 Exosomes as biomarker

According to Biomarker Definitions Working Group in National Institutes of Health, biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In other words, biomarker is the indicator of change and therefore fluctuates as a function of time and biological influence (Müller, 2012). Exosomes are not only specifically targeted to recipient cells

to exchange proteins and lipids or to trigger downstream signaling events, but also deliver specific nucleic acid cargo. The unique function of exosomes might be specific interaction with a target recipient cell, enabling cell-to-cell communication, putatively between widely separated locations in the body (Vlassov et al., 2012), which might be useful as biomarker for diagnosis and therapeutic purposes. Exosomes presented in biofluids most likely represent a mixture derived from various cell types (Keller et al., 2011) and can be easily recovered (Van Niel, 2006). Based on these properties, biofluids-derived exosomes can be served as a potential biomarker in many studies (Chaput & Théry, 2010; Kalra et al., 2013; Keller et al., 2011).

1.2 Adipose tissues and obesity

1.2.1 Adipose tissues

Adipose tissue is a heterogeneous tissue composed of adipocytes, and various microvascular and immune cells in its stromal vascular fraction (SVF) (Hajer, et al., 2008; Lafontan, 2014). Generally, adipose tissues are divided into two main compartments: central (subcutaneous upper abdominal and visceral fat) and peripheral (hip and gluteal-femoral fat) (Lafontan, 2014). When excessive energy intake occurs beyond body needs, triglyceride will be stored as lipid droplets in white adipocytes. White adipocytes compose of one or few large lipid droplets that occupy almost all the entire cell volume and thereby determine the cell size (Müller, 2012), and are major sites of energy storage and important for energy homeostasis (Yamauchi & Kadowaki, 2013). Adipose tissues also produce hormones and cytokines involved in glucose metabolism (e.g. adiponectin, resistin), inflammation (e.g. TNF-α, IL-6), coagulation (PAI-1), blood pressure (e.g. angiotensinogen, angiotensin II), and feeding behavior (leptin), thus affecting metabolism and function of many organs and tissues including muscle, liver, vasculature, and brain (Hajer et al., 2008). Expression of these hormones and cytokines are related to nutritional state. Improper expression of hormones and cytokines is resulting in metabolic syndrome, as a consequence of adipose tissue excess or deficiency. This study focuses on adiponectin, leptin, and

resistin, which are exclusively produced by adipose tissues as described in the following details.

1.2.1.1 Adiponectin

Adiponectin (ACRP30, AdipoQ, apM1 or GBP28) is a 30-kDa protein, containing a collagen-like fibrous domain at N-terminal and C1q-like globular domain. The most basic form of adiponectin is trimer, associated through disulfide bonds with in collagenous domains of each monomer to form bouquet-like higher order structures (Trujillo & Scherer, 2005) (Fig. 3). The higher complex structures include low-molecular-weight (LMW) trimer, middle-molecular-weight (MMW) hexamer, and high-molecular-weight (HMW) 12- to 18-mer (Yamauchi & Kadowaki, 2013), and these complexes are stable both *in vivo* and *in vitro* (Pajvani et al., 2002). Adiponectin is produced and secreted exclusively from white adipose tissue (WAT) and circulates at relatively high concentration, 0.5-30 μg/ml in human (Budak et al., 2006; Meier & Gressner, 2004). Full-length adiponectin (both LMW and HMW complex forms) was found in human plasma and serum, accounted for 0.01% of total serum and plasma protein (Okamoto et al., 2006; Trujillo & Scherer, 2005). Mouse serum contains both full-length and small amount of shorter 25 kDa globular domain, accounted for 0.05% of total serum protein. A proteolytic cleavage product containing the globular domain of adiponectin also circulates at physiologically significant levels and has biological activity than native adiponectin in some studies (Beltowski, 2003; Kershaw & Flier, 2004).

Synthesis and secretion of adiponectin are regulated by several mechanisms (Beltowski, 2003). The adiponectin gene expressed exclusively in adipocytes has been reported to be regulated by transcriptional factors including CCAAT-enhancer-bindingproteins (C/EBPs), sterol regulatory element binding protein 1c (SREBP1c), and peroxisome proliferator-activated receptor γ (PPARγ) (Yamauchi & Kadowaki, 2013). Yamauchi and Kadowaki (2013) summarized the regulatory mechanisms of adiponectin formation (Fig. 4). Sirtuin 1 (NAD-dependent deacetylase sirtuin 1 or SIRT1) has been reported to deacetylate Lys268 and Lys 293 of PPARγ and to cause selective PPARγ modulation, leading to upregulation of adiponectin. There is an abundant pool of properly folded adiponectin in the secretory pathway

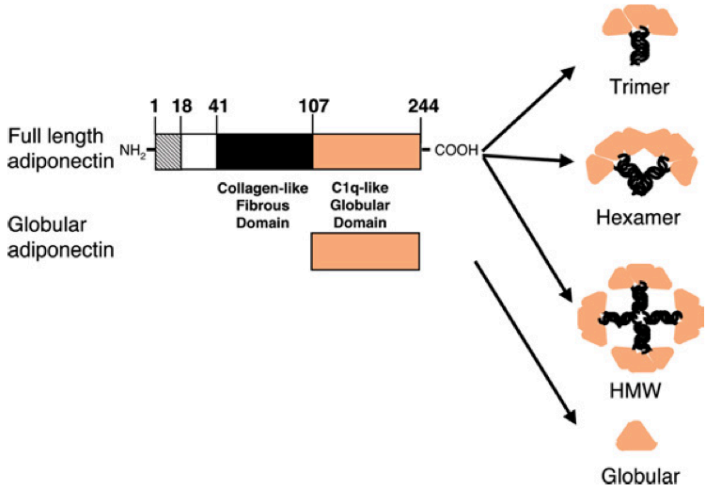


Fig. 3 : Domains and structure of adiponectin (Okamoto et al., 2006)

through thiol-mediated retention, and that adiponectin is covalently bound to endoplasmic reticulum (ER) chaperone ERp44. Another ER chaperone, ER oxidoreductase Ero1-Lα, plays a critical role in the release of adiponectin from ERp44, and that these chaperones play a major role in the assembly of HMW adiponectin. Hyperinsulinemia, oxidative stress and inflammation observed in obesity have been reported to reduce HMW adiponectin, whereas thiazolidinediones (TZDs) and caloric restriction have been reported to increase HMW adiponectin (Yamauchi & Kadowaki, 2013). In cell culture, insulin stimulates adiponectin gene expression and secretion in 3T3-L1 adipocytes. In human adipocytes isolated from visceral adipose tissue, insulin and insulin-like growth factor-1 (IGF-1) also increase adiponectin synthesis (Beltowski, 2003).

Adiponectin mainly functions in adipose tissue metabolism via autocrine signaling on adiponectin receptor, named AdipoR1 and AdipoR2. AdipoR1 and AdipoR2 serve as receptors for adiponectin in regulation of glucose and lipid metabolism, inflammation, and oxidative stress in metabolic syndrome via intracellular signal transduction mechanisms. AdipoR1 is a high-affinity receptor for globular adiponectin and low-affinity receptor for full-length adiponectin and involved in activation of AMP-activated protein kinase (AMPK); whereas AdipoR2 is an intermediate-affinity receptor for both globular and full-length adiponectin and

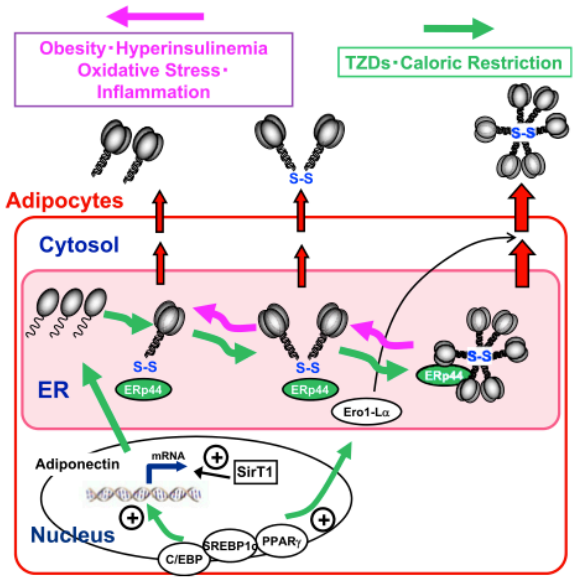


Fig. 4 : Regulatory mechanisms of adiponectin formation (Yamauchi & Kadowaki, 2013)

involved in activation of PPARα (Fu, 2014; Yamauchi & Kadowaki, 2013). Apart from adipose tissues, adiponectin also regulates metabolisms in other organs by paracrine/endocrine signaling on AdipoR1/R2 receptors. AdipoR1 is abundantly expressed in skeletal muscle, while AdipoR2 is predominantly expressed in liver. Moreover, adiponectin can interact with a putative receptor, T-cadherin, but the biological significance of this non-transmembrane receptor is not clear yet. T-cadherin receptor probably acts as a co-receptor to exert its functions with the integrin pathway or AdipoR1/AdipoR2 receptors (Fu, 2014) (Fig. 5).

Clinical studies have been reported the relationship between adiponectin and metabolic diseases and inflammation states (Kershaw & Flier, 2004). Adiponectin has a negative correlation with body weight, fat mass, and insulin level. Decrease in adiponectin concentration leads to insulin resistance, lipid synthesis, increase in TNF-α level, resulting in type 2 diabetes mellitus, obesity, inflammation, and related diseases such as cardiovascular diseases, hypertension, dyslipidemia. Moreover, hypoadiponectinemia is also related with cancers, sleep apnea, non-alcoholic fatty liver disease, gastritis and gastroesophagal reflux disease, pancreatitis, osteoporosis, inflammatory bowel disease and defective reproductive

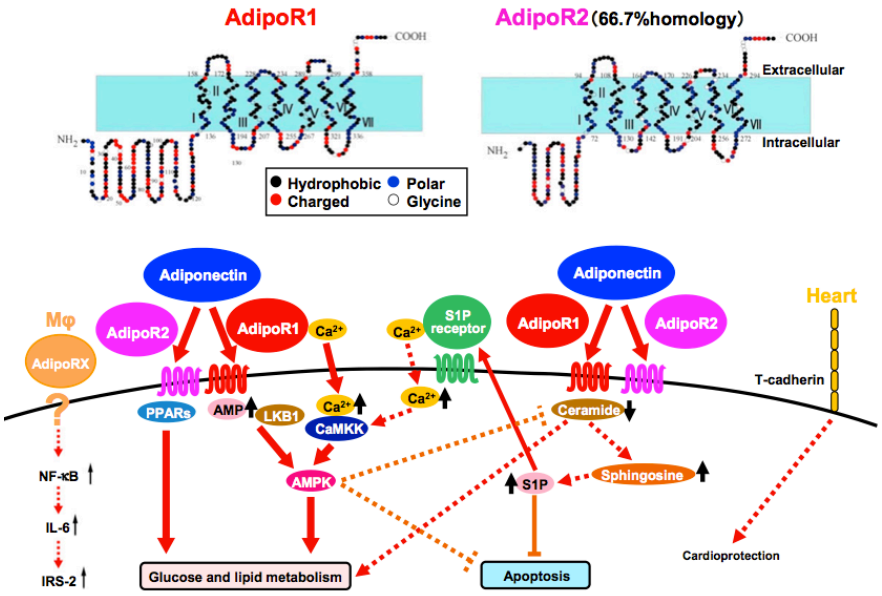


Fig. 5: Structure and function of adiponectin receptors (Yamauchi & Kadowaki, 2013)

system (Beltowski, 2003; Budak et al., 2006; Kishida, et al., 2014; Meier & Gressner, 2004). On the other hand, excessive adiponectin level, or hyperadiponectinemia, is also reported to be associated with cardiac dysfunction, pulmonary disease, and chronic kidney disease (Kishida et al., 2014). Therefore, adiponectin holds a great promise as a biomarker in clinical practices (Kishida et al., 2014; Okamoto et al., 2006; Trujillo & Scherer, 2005)

1.2.1.2 Leptin

Leptin is a 16-kDa non-glycosilated polypeptide of 146 amino acids as a product of *ob* gene (Budak et al., 2006; Meier & Gressner, 2004). Leptin is predominantly produced by differentiated adipocytes and plays important roles in food intake and energy expenditure. Leptin expression and secretion are also regulated by a variety of factors. For example, leptin is increased by insulin, glucocorticoids, TNF-α, estrogens, and C/EBPα and decreased by β3-adrenergic activity, androgens, free fatty acids, growth hormones, and PPAR-γ agonists (Kershaw & Flier, 2004). Circulating leptin acts on hypothalamus crossed blood-brain barrier through the Janus

kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Amita Yadav et al., 2013), mitogen-activated protein kinase, phosphatidyl-inositol 3'-kinase (PI3K) and AMPK (Lafontan & Viguerie, 2006). In serum, leptin is found as free-and protein-bound forms (Budak et al., 2006). There are six isoforms of leptin receptor (Ob-Ra,b,c,d,e, and f), which share the same extracellular domain but differ in the length of intracellular region or differ in transmembrane domain (Budak et al., 2006). Ob-Rb has the longest intracellular domain, has the highest concentration in hypothalamus and mediates weight reducing property of leptin (Meier & Gressner, 2004; Kershaw & Flier, 2004). Ob-Re has been shown as the majority of serum leptin-binding activity and plays a key role in regulation of bioactivity of leptin (Budak et al., 2006). Leptin concentration increases direct proportional to fat mass and nutritional status, and primary role of leptin is to serve as a metabolic signal of energy sufficient rather than excess and acts together with insulin to inhibit food intake at hypothalamus level (Budak et al., 2006; Kershaw & Flier, 2004). Leptin level rapidly decreases under caloric restriction and weight loss, being associated with adaptive physiological responses to starvation including increased appetite and decreased energy expenditure (Kershaw & Flier, 2004). *Ob/ob* mutant mice produce a defective, non-functional leptin that results in a phenotype of obesity and sterility because leptin cannot signal to the brain (Budak et al., 2006).

1.2.1.3 Resistin

Resistin is a 12.5-kDa member of cysteine-rich secretory protein family called "resistin-like molecules" (RELM) or "found in inflammatory zone" (FIZZ) (Beltowski, 2003). Resistin is expressed exclusively in white adipose tissues and involved in the development of obesity and insulin resistance via increased expression of gluconeogenic enzymes in liver and decreased activity of AMPK and insulin receptor substrate (IRS)-2 expression (Beltowski, 2003; de Oliveira Leal & Mafra, 2013). High-fat diet-fed, mutation of leptin gene (*ob/ob*) and mutation of leptin receptor gene (*db/db*) mice are associated with increased circulating resistin concentration (Budak et al., 2006; Meier & Gressner, 2004). Resistin circulates in form of dimeric protein (Meier & Gressner, 2004). Resistin increases blood glucose and induces insulin resistance in mice, while anti-resistin antibody reduces blood

glucose and improves insulin sensitivity. Effect of insulin on the regulation of resistin expression are still controversial (Beltowski, 2003; Kershaw & Flier, 2004). *In vitro*, insulin downregulates resistin expression, whereas insulin treatment in obese Zucker rats and in streptozotocin-induced diabetes mice shows stimulatory effect (Beltowski, 2003). Treatment of dexamethazone upregulates resistin expression in adipocyte, indicating important role of glucocorticoids (Beltowski, 2003). The observed improvement in glucose homeostasis in mice lacking resistin is associated with decreased hepatic gluconeogenesis. This effect is mediated at least in part via increased activity of AMPK and decreased expression of gluconeogenic enzymes in the liver. Nevertheless, human resistin shares only 64% homology with murine resisitin and is expressed at vey low levels in adipocytes. Therefore, these data support function of resistin in glucose homeostasis during fasting in rodents, although the similar function of resistin in humans remains to be determined (Kershaw & Flier, 2004). In fact, some studies support the association between resistin and obesity or insulin resistance. Resistin was reduced following hypocaloric diet- and exercise-induced weight loss. Its expression was also reduced after bariatric surgery. As such, it is likely that human resistin is associated with obesity and is responsive to change in adiposity (de Oliveira Leal & Mafra, 2013).

1.2.2 Obesity

1.2.2.1 Characteristics of obesity

Obesity can be characterized by low-grade chronic inflammation of adipose tissues. Adipose tissue expansion occurs when adipocyte numbers and size increase, which is known as hyperplasia and hypertrophy, respectively (Sano et al., 2014). Hypertrophy, to a certain degree, is a characteristic of all overweight and obese individuals. Hyperplasia, however, is correlated more strongly with obesity severity and is most marked in severe obese individuals (Moreno-Navarrete & Fernández-Real, 2011). Adipose tissues compose of small (diameter <50 μm) and large (diameter >100 μm) adipocytes, which accompanied by profound difference in lipid storage, expression of major genes of lipid and glucose metabolism and secretory function (Müller, 2013). The concept of body fat distribution may be regulated partly through differences in early adipogenesis. Dimension of adipocytes during obesity may result

in their disruption due to physical and geometrical restraints (Müller, 2013). Paradigm of insulin resistance in obese subjects relies on drastically increased numbers of large adipocytes. Adipocyte hypertrophy exhibits higher plasma insulin levels and lower insulin sensitivity compared to adipocyte hyperplasia (Müller, 2013). Obesity leads to adipose tissue dysfunction associated with combined effect of change in lipid storage capacity and mobilization, recruitment of macrophages and T cells, and changes in adipokines level. As mentioned above, fatty acids are stored in adipose tissues as source of energy. They also serve as secondary messengers implicated in hepatic and skeletal muscle fatty acid-induced insulin resistance, signal nutrient mediation to the central nervous system, and have central effects on insulin action (Lafontan, 2014). Moreover, fatty acids are involved in lipid storage defect in adipose tissues. Caveolae are small invagination of plasma membranes, composes of cholesterol, sphingolipids, and specific protein cavin and caveolin (Lafontan, 2014) (Fig. 6). Caveolae represent 30-50% of adipocyte surface area and are involved in sensing membrane tension during swelling, which is important for adjusting size of growing adipocytes and are also critical for insulin signaling (Lafontan, 2014). Decrease in membrane cholesterol/caveolae means adipocyte's size increase, and this is a key regulator of inflammatory response in accordance to adipocyte dysfunction (Lafontan, 2014).

1.2.2.2 Dysfunctional adipose tissues caused by obesity

During excessive calorie intake, hypertrophied adipocytes secrete large amount of cytokines involved in inflammatory response including monocyte chemoattractant protein-1 (MCP-1)/ chemokine (C-C motif) ligand 2 (CCL2), and TNF-α. Large adipocytes release fatty acids which can bind to macrophage toll-like receptor-4 (TLR-4), resulting in NF-κB activation and TNF-α production consequently (Hajer et al., 2008). Monocytes from blood migrate into adipose tissues and differentiate into macrophages by MCP-1/CCR2 and intracellular adhesion molecule-1 (ICAM-1) stimulation (Hajer et al., 2008; Holvoet, 2012). Meanwhile, TNF-α stimulates $CD4^+$Th1 cells and $CD8^+$effector T cells accumulation. $CD4^+$Th1 cells play a role in insulin resistance while $CD8^+$effector T cells paly a role in macrophage accumulation, recognition and elimination of damaged and dysfunctional cells in adipose tissues (Lolmede et al., 2011; Holvoet, 2012; Lafontan, 2014). Besides, adipose

a) b)

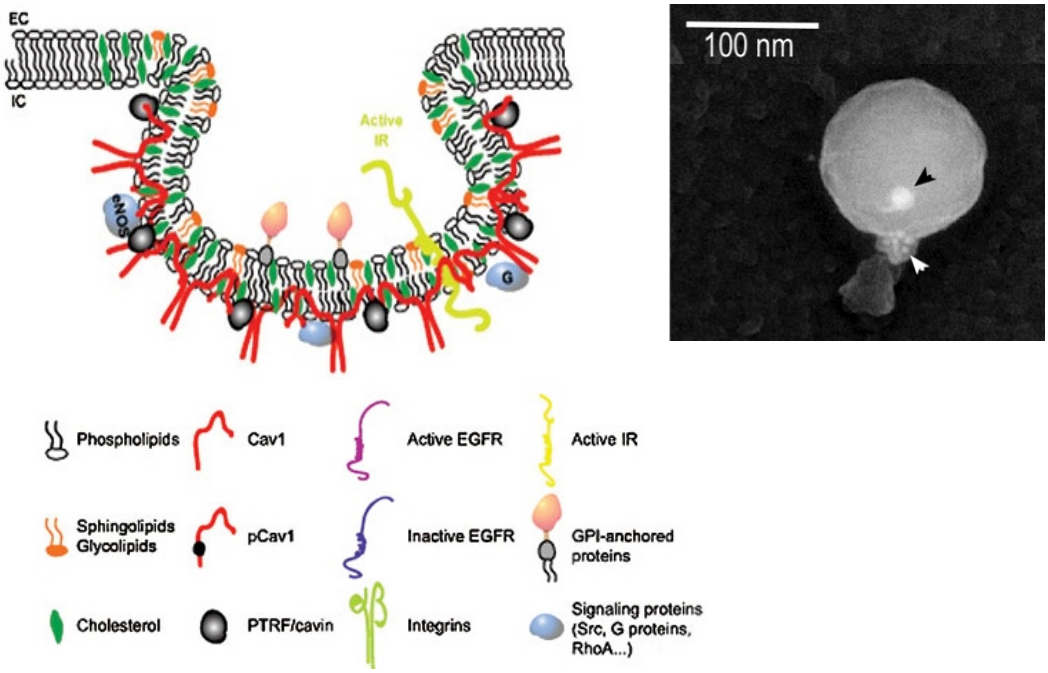


Fig. 6 : Structure of caveolae. a) Caveolae compose of cholesterol-enriched domains where receptors and signaling protein (including GPI-anchored proteins, cavin and caveolin) accumulate and signal (Boscher & Nabi, 2012). b) Electron micrograph of the inner surface of the plasma membrane from a human adipocyte. A single caveola attached to the plasma membrane is seen by immunogold labelling of the insulin receptor (large gold particle, black arrowhead) and caveolin-1 (small gold particles, white arrowhead) (Stralfors, 2012).

tissue inflammation and decrease in insulin sensitivity are also associated with $CD4^+$ Treg cells reduction (Lolmede et al., 2011; Holvoet, 2012). Activated T cells and chemokines (IL-6 and TNF-α) promote macrophages from anti-inflammatory state (M2) to pro-inflammatory state (M1) (Torres-Leal et al., 2012). Recruitment of macrophages in obese adipose tissues can be accounted up to 30-50% in total adipose tissue cell population as compare to 10% of macrophages population in lean adipose tissue cell population (Kaliman & Parrizas, 2011). Interaction between dysfunctional adipose tissues, activated T cells, and chemokines leads to dysregulation of adipokines (Holvoet, 2012), by overexpression of resistin, leptin and reduction of adiponectin

levels (Holvoet, 2012; Torres-Leal et al., 2012). Another mechanism of dysfunctional adipose tissues leading to insulin resistance is involved in oxidative stress and cell apoptosis (Holvoet, 2012). The summary of dysfunctional adipocytes during obesity is shown in fig. 7.

1.3 Aim of study

Exosomes harbor a wide variety of proteins, lipids, mRNA, and microRNA, which can be transferred to another cell, and are implicated in intercellular communication by transferring molecules (Johnstone et al., 2001; Skog et al., 2008; Valadi et al., 2007). Previous studies have identified exosomes in the culture supernatant of mouse adipose tissues (Deng et al., 2009), rat primary adipocytes (Müller et al., 2009a; Müller et al., 2009b; Müller et al., 2011), and mouse adipocyte cell line 3T3-L1 (Ogawa et al., 2010; Sano et al., 2014). Deng et al. (2009) isolated exosomes from the culture supernatant of visceral adipose tissue excised from mice and showed that injection of the exosomes derived from diet-induced or genetically (leptin deficient *ob/ob*) obese mice into wide type lean mice results in macrophage activation and insulin resistance (Deng et al., 2009). Müller et al. (2009a) found that rat primary adipocytes release exosomes which may transfer lipogenic and/or lipolytic information between large and small adipocytes (Müller et al., 2011; Müller et al., 2009a; Müller et al., 2009b). More recently, Sano et al. (2014) isolated exosomes from the culture supernatant of differentiated 3T3-L1 cells and demonstrated that exosomes isolated from cell cultured under hypoxic conditions are enriched in enzymes related to lipogenesis and promote lipid accumulation in recipient 3T3-L1 adipocytes (Sano et al., 2014). Adipocyte-derived exosomes contain 7,000 mRNAs and 140 miRNAs, and the majority of these are adipocyte-specific and abundantly expressed in the donor adipocytes. Moreover, adipocyte-specific transcripts for adiponectin and PPARγ2 are present in the adipocyte-derived exosomes (Rome, 2013). These findings suggest that adipocyte-derived exosomes play a role in cell-to-cell communication during the development of metabolic diseases *in vivo*. However, the characteristics and function of exosomes released from adipocytes remain to be elucidated. Clearly, exosomes released from adipocytes exists in circulation. In addition, because the composition of exosomes is heterogenic, depending on the cellular origin of exosomes, adipocyte-

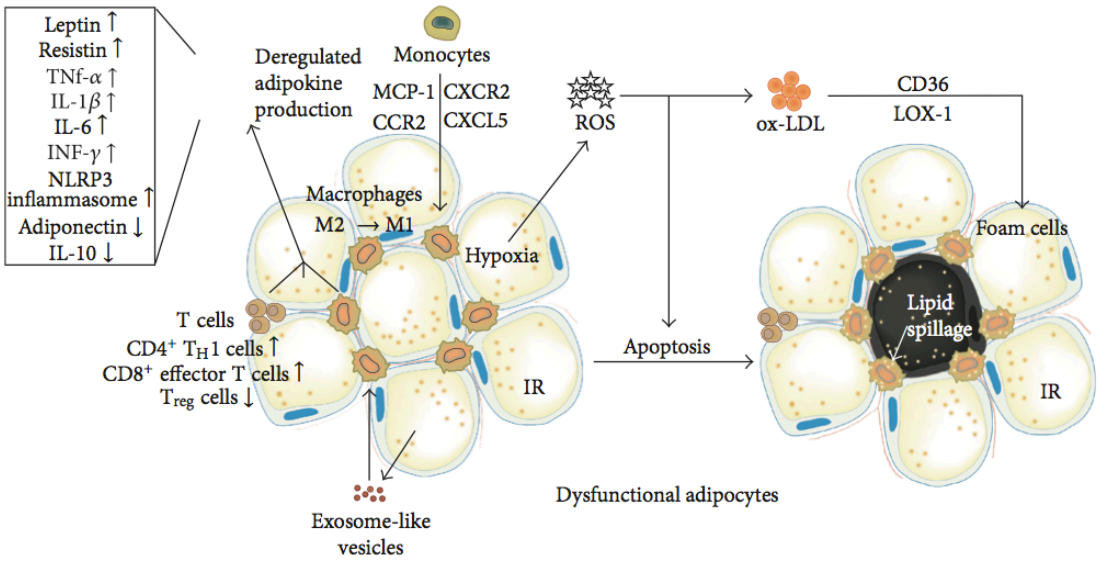


Fig. 7 : Changes in adipocyte during obesity lead to insulin resistance (Holvoet, 2012)

derived exosomes could be accompanied by molecules produced specially in adipocytes.

In this context, this study postulated that such molecules associated with exosomes in the serum could be markers for adipocyte-derived exosomes *in vivo*. This study particularly focused on secretory protein produced specifically in adipocytes, namely adipokines including adiponectin, leptin, and resisitin.

CHAPTER II
MATERIALS AND METHODS

2.1 Animals and diets

All study protocols were approved by the Animal Use Committee of the Hokkaido University Research Faculty of Agriculture (approval no. 08-0139). Animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Male C57BL/6J JmsSlc (B6), C57BL/6J HamSlc-+/+ (+/+), and C57BL/6J HamSlc-*ob/ob* (*ob/ob*) mice (5 weeks old) were purchased from Japan SLC and housed in a plastic cages in a controlled environment (23±2ºC) room under a 12-hr light-dark cycle. They were allowed free access to water and standard chow diet (MR stock; Nosan Corporation, Japan) for 1 week. In a separate experiment, male B6 mice (5 weeks old) were fed with normal-fat diet (NFD) (10% kcal from fat, D12450B, Research Diets, USA) or high-fat diet (HFD) (60% kcal from fat, D12492, Research Diets) for 4 months.

2.2 Cell culture

RAW 264.7 macrophages were maintained in DMEM (high-glucose, pyruvate, 12800-017, Gibco, USA) supplemented with 100 unit/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, and 10% (v/v) fetal calf serum (basal medium) in an incubator with CO_2 5% at 37°C. For experiment, RAW 264.7 cells ($5x10^4$ cells/100 μl) were cultured in each well of 96-well plate (353072, BD falcon, USA) and incubated at 37°C. After 24 hr, the medium was replaced with 0.5% FCS DMEM and incubated at 37°C overnight. Medium was then replaced by 50 μl of 0.5% FCS DMEM, supplemented with or without 5 or 10 μg/ml of exosome samples. The cells were allowed to incubated at 37°C for 6 hr before adding 100 ng/ml LPS in 0.5% FCS DMEM and incubated at 37°C. After 24 hr, conditioned medium was collected and kept at -20°C for ELISA analysis.

2.3 Exosome isolation by differential centrifugation

Mice were deprived of food overnight, and total blood was obtained from carotid artery under diethyl ether anesthasia. Exosomes were isolated by differential centrifugation according to Théry et al. (2006) with modification. Serum samples (100 µl) were removed cell debris by centrifugation at 2,000×g for 10 min at 4°C, followed by 10,000×g for 10 min at 4°C (3500 model, Kubota, Japan). The serum samples were then diluted with 400 µl PBS and ultracentrifuged at 100,000×g for 2 hr at 4°C with S100AT4 rotor (CS100GXL, Hitachi, Japan). The pellet was washed again with 500 µl PBS and subjected to ultracentrifuged at 100,000×g for 2 hr at 4°C. The post-ultracentrifuge pellet was suspended in the same volume of PBS as the original serum samples. This suspension was referred to as the exosome fraction. The protein concentration in the serum and exosomes fraction was determined by a BCA protein assay (Pierce, USA) according to the manufacturer's instructions. Samples were stored at -80 °C until use.

2.4 Exosomes purification by a discontinuous Iodixanol (OptiPrep™)–sucrose density gradient

Exosomes were further separated by discontinuous OptiPrep™ gradient ultracentrifugation according to Mathivanan et al. (2010), with slight modification. In brief, the exosome fraction (500 µl) was overlaid on a discontinuous OptiPrep™-sucrose gradient (50, 25, 12.5, and 6% Iodixanol concentration from OptiPrep™ solution (Axis-Shield PoC, Norway) in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) and ultracentrifuged for 18 hr at 100,000×g, 4°C in a Beckman SW40Ti rotor (L8-80M, Beckman Coulter). Fractions (1 ml) were collected from the top of the gradient and diluted with 2 ml of 10 mM Tris-HCl, pH 7.4, followed by ultracentrifugation for 3 hr at 100,000×g, 4°C. The pellet in each fraction was resuspended in 100 µl PBS and then western blotted.

The density of each fraction was determined by absorbance at 340 nm using a duplicate parallel discontinuous OptiPrep™ gradient overlaid with 500 µl of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4. Standard sucrose-Optiprep™ solution (6,

12, 24, 32, 36, 44, and 50% Iodixanol in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) and 12 fractions obtained from sucrose-overlaid samples were 2 fold-diluted with DW 2 times. A 100 μl of diluted standard and samples were transferred to 96-well plate for absorbance measurement. The absorbance was measured using Synergy Mx microplate reader (BioTek Instruments, USA). A solution of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 was used as a blank. The density of sucrose-Optiprep^{TM} standard was referred to the manufacture's (Axis-Shield PoC) application sheet (Appendix D).

2.5 Treatment of exosomes with proteinase K and Triton X-100

Exosome fraction was treated with either 30 μg/ml proteinase K, 0.5% Triton X-100, or a combination of both proteinase K and Triton X-100 at 37°C for 5, 15, or 30 min, after which 3x SDS-PAGE loading buffer compose of 195 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 30% (w/v) glycerol, and 15% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol blue was added. After boiling for 3 min, samples were subjected to SDS-PAGE and then western blotted.

2.6 SDS-PAGE and western blot analysis

Samples were separated by SDS-PAGE under reducing and non-reducing conditions. For reducing conditions, samples were incubated in 3x sample buffer (195 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 30% (w/v) glycerol, and 15% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol blue) at 100°C for 3 min. The samples were loaded in 12-14% SDS-PAGE gel. The gel was run in 1x running buffer, composed of 250 mM of Tris, 1,920 mM of glycine, and 1% (w/v) SDS, at 150 V for approximately 1.5 hr. Then, the gel and Optitran nitrocellulose membrane (BA-S-85, 0.45 μm, GE Healthcare Life Sciences, UK) were soaked in 12% gel transfer buffer, composed of 250 mM of Tris and 1,920 mM of glycine. The gels were transferred to nitrocellulose membrane with semi-dry transfer system (ATTO, Japan) at 170 mA for 1 hr. The membranes were washed wih 0.9% NaCl solution and air dry before kept at 4°C or blocked in 0.5% (w/v) BSA in Tris-bufferred saline with 0.1% (v/v) Tween 20 (TBS-T) at RT for 1 hr. Membranes were then probed with mouse anti-adiponectin (Enzo Life Science, USA; 1:3,000-5,000), rat anti-CD63 (Biolegend, USA; 1:500), rabbit anti-Leptin (BioVendor, Czech Republic; 1:500), and rat anti-resistin (Enzo Life

Science; 1:500) overnight at 4°C. After washing with TBS-T with shaking at 60 rpm for 5 min 3 times, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG1 (Santa Cruz Biotechnology, USA; 1:8,000-10,000), or horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology; 1:2,000), horseradish peroxidase-conjugated goat anti-rat IgG (Santa Cruz Biotechnology; 1:2,000) at RT for 1 hr. The blots were washed with TBS-T with shaking at 60 rpm for 5 min 3 times and were visualized by chemiluminescense (ECL Plus Western blotting detection reagents, GE Healthcare, USA). The images were analyzed by LumiVision PRO 400EX (Aisin Seiki Co., Ltd., Japan)

For non-reducing conditions, samples were incubated in 3x sample buffer without β-mercaptoethanol. Samples were not boiled and then loaded in 4-15% gradient SDS-PAGE gel and transferred to nitrocellulose membrane, followed by western blotting as described above.

2.7 ELISA

Serum, conditioned media and exosome samples were analyzed by ELISA for adiponectin (mouse Adiponectin/ Acrp30, R&D Systems, USA), leptin (Mouse Leptin ELISA Kit, Morinaga Institute of Biological Science, Inc., Japan), resistin (Mouse Resistin Quantikine ELISA Kit, R&D Systems), TNF-α (eBioscience), and IL-6 (eBioscience) according to the manufacture's instruction. The absorbances were measured by Synergy Mx microplate reader (BioTek Instruments).

2.8 Statistical analysis

Results are presented as means ± SEM. Student's *t*-test was used to compare mean values. Data analysis was performed using GraphPad Prism for Macintosh (version 6; GraphPad Prism Software, USA). *P* values of <0.05 were considered to be statistically significant.

CHAPTER III
Results

3.1 Serum adiponectin is partially associated with exosomes

Samples containing the same amount of protein in the serum, exosome fraction, and supernatant from the initial ultracentrifugation of serum were separated by SDS–PAGE, stained with Coomassie brilliant blue (CBB) (Fig. 8A) and western blotted with a probe to an antibody against CD63, a typical marker protein of exosomes (Vlassov et al., 2012). The probe signal was observed in all three samples, and the intensity was higher in the exosome fraction than in the serum or supernatant (Fig. 8B). Next, we separated samples having the same volume of serum, exosome fraction, and supernatant. On western blotting with anti-adiponectin antibody, a probe signal was observed in all three fractions, with a lower intensity in the exosome fraction (Fig. 9). In addition, a leptin probe signal was observed in the serum and supernatant, but not the exosome fraction. Furthermore, a resistin probe signal was observed in the serum and supernatant, but only a faint signal was detected in the exosome fraction. Although the signal intensity for both CD63 and adiponectin was unchanged by Triton X-100 treatment, proteinase K treatment quenched the CD63 signal (Fig. 10). Similarly, the signal intensity for adiponectin was lowered by proteinase K treatment, though a faint signal remained. However, the combination of proteinase K and Triton X-100 completely quenched the adiponectin signal. On western blots of fractions from the ultracentrifugation on a discontinuous gradient, signals for adiponectin and CD63 were detected at a density of 1.17 g/ ml (fraction 10) (Fig. 11). In addition, weaker signals for adiponectin and CD63 were also observed at a density of 1.07–1.08 g/ml (fractions 4 and 5).

3.2. High-molecular weight adiponectins are associated with exosomes

Adiponectin exists in the circulation as multimeric complexes including a low-molecular-weight (LMW, 67 kDa), middle-molecular-weight (MMW, 136 kDa) and high-molecular-weight (HMW, >300 kDa) forms (Vlassov et al., 2012) .

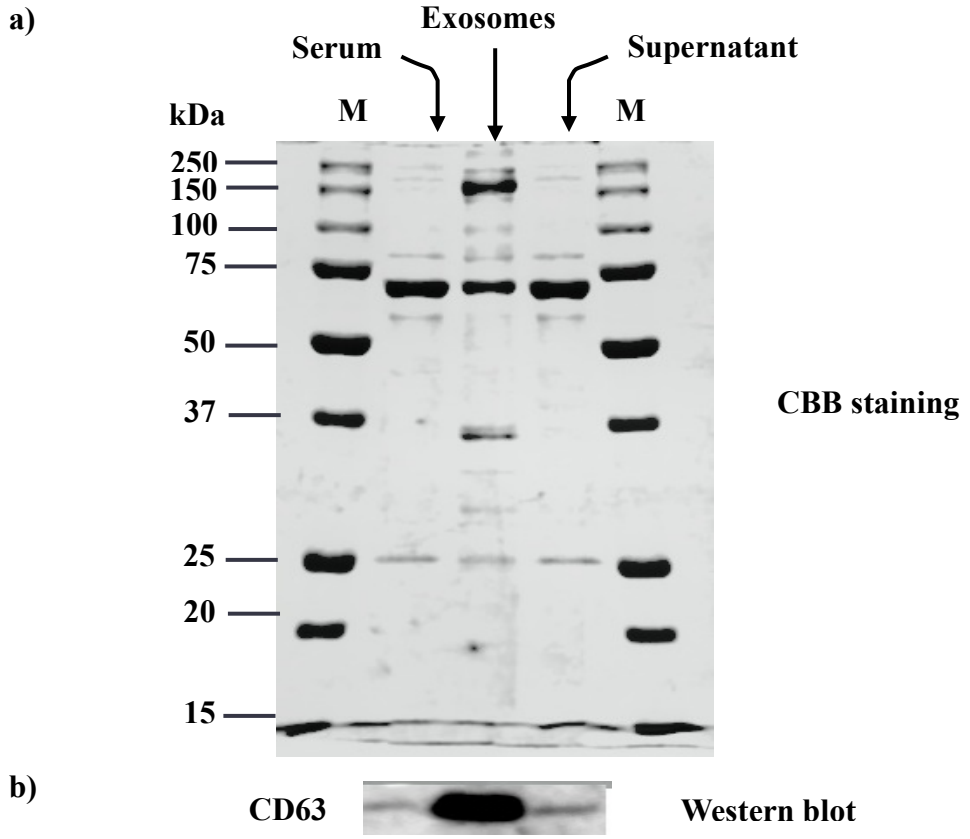


Fig. 8 : CBB staining and western blotting of CD 63. a) Coomassie brilliant blue staining of SDS-PAGE gel of serum, exosomes, and supernatant of C57BL/6 mice. b) Western blot probed with antibody to CD63

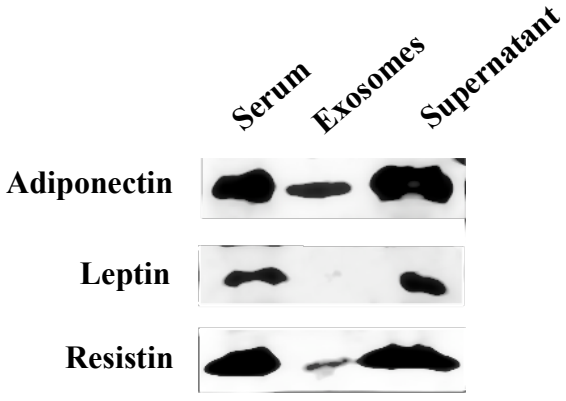


Fig. 9 : Western blotting of adiponectin, leptin, and resisitin. Samples in which the original serum volumes were the same were applied in each lane, electrophoresed under reducing conditions, and then probed with antibodies to adiponectin, leptin, and resistin.

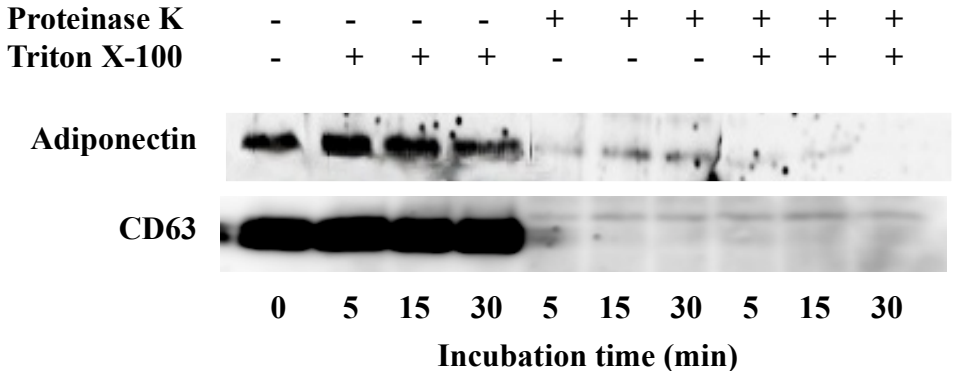


Fig. 10 : Effect of proteinase K and Triton X-100 on adiponectin and CD63 in the exosome fraction prepared from C57BL/6 mouse serum. The exosome fraction was treated either with 30 μg/ml proteinase K, 0.5% Triton X-100, or a combination of both at 37 °C for 5, 15, or 30 min. Samples were electrophoresed under reducing conditions and then probed with antibodies to adiponectin and CD63.

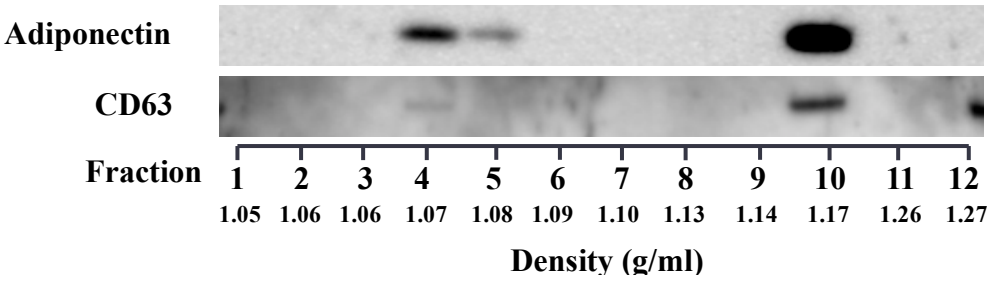


Fig. 11 : Density gradient separation of exosomes prepared from B6 mouse serum. The exosome fraction was separated by ultracentrifugation on a discontinuous OptiPrep™ gradient and electrophoresed under reducing conditions, then probed with antibodies to adiponectin and CD63.

When samples with the same adiponectin concentration measured by ELISA were separated by SDS–PAGE under nonreducing conditions followed by western blotting, the MMW form was predominant in the serum and supernatant, whereas the HMW form was predominant in the exosome fraction in NFD-fed and HFD-fed mice (Fig. 12). Under the same condition, samples from +/+ and *ob/ob* mice also showed that HMW form was predominantly found in exosomes fraction and MMW form was mainly found in serum and supernatant part (Fig. 13).

3.3. Adiponectin is decreased in the serum and exosomes in obese mice

Although leptin was not detected in the exosome fraction by ELISA, the concentration of adiponectin and resistin in the exosome fraction was 154 ± 28 and 0.36 ± 0.05 ng/ml, which respectively account for 4.4 ± 1.0 and 0.9 ± 0.1% of the proteins in the serum (Table 1). Compared to +/+ lean mice, the serum concentration of total protein in genetically obese *ob/ob* mice was higher, while the serum concentration of adiponectin was lower (Table 2). Similarly, total protein concentration in the exosome fraction was higher in *ob/ob* mice than in +/+ mice. In contrast, there were no differences in adiponectin concentration in the exosome fraction between the two groups. In the exosome fraction, the ratio of adiponectin to total protein was significantly lower in *ob/ob* mice (0.0157 ± 0.0031%) than in +/+ mice (0.0315 ± 0.0018%). The concentration of adiponectin in the exosome fraction in +/+ and *ob/ob* mice accounts respectively for 1.9 ± 0.4 and 2.1 ± 0.5% of the adiponectin in the serum. In addition, HFD-fed obese mice had a lower serum concentration of adiponectin than NFD-fed lean mice, whereas total protein concentration in the serum was the same for the two groups (Table 3). In the exosome fraction, there were no differences in either total protein or adiponectin concentration between the groups, while the ratio of adiponectin to total protein was significantly lower in HFD-fed obese mice (0.0515 ± 0.0098%) than in NFD-fed lean mice (0.0766 ± 0.0062%). The concentration of adiponectin in the exosome fraction in NFD-fed and HFD-fed mice accounted respectively for 11.3 ± 1.3 and 10.9 ± 2.2% of adiponectin in the serum.

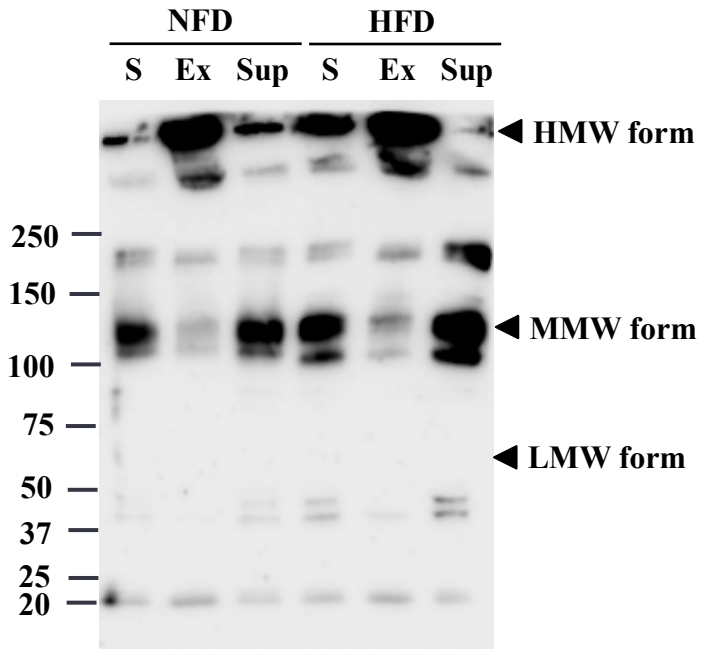


Fig. 12 : Western blotting of adiponectin under nonreducing, unheated conditions of NFD- and HFD-fed mice. The exosome fraction was prepared from serum (S), exosome (Ex), and supernatant from exosome isolation (Sup) of C57BL/6 mouse fed with either normal fat-diet (NFD) or high-fat diet (HFD). Samples with the same adiponectin concentration were applied in each lane and probed with anti-adiponectin antibody.

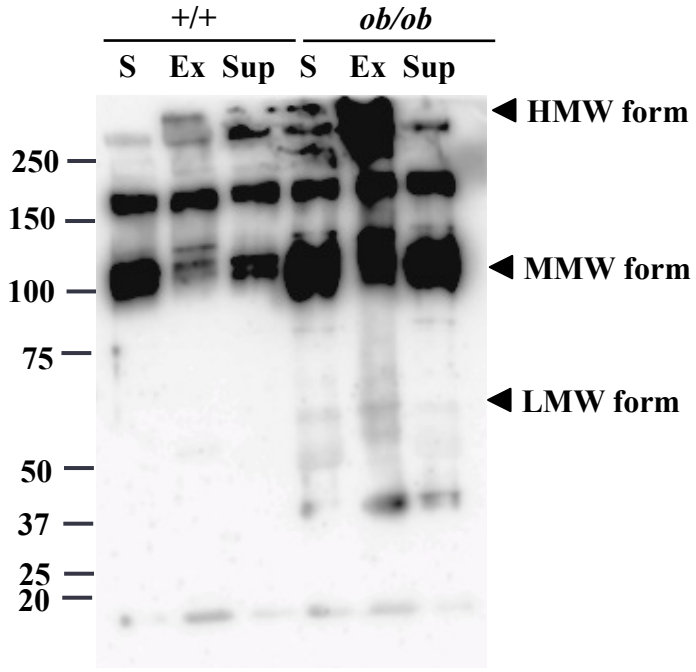


Fig. 13 : Western blotting of adiponectin under nonreducing, unheated conditions of +/+ and *ob/ob* mice. The exosome fraction was prepared from serum (S), exosome (Ex), and supernatant from exosome isolation (Sup) of control (+/+) and genetically obese mouse (*ob/ob*). Samples with the same adiponectin concentration were applied in each lane and probed with anti-adiponectin antibody.

Table 1 : Concentration of total protein and adipokines in serum and exosome fraction of C57BL/6 mice

	Serum	**Exosome fraction[1]**
Total protein (mg/ml)	48.1±0.8	0.47±0.06
Adiponectin (ng/ml)	3,745±241	154±28
Leptin (ng/ml)	5.89±0.12	Not detectable
Resistin (ng/ml)	39.73±1.55	0.36±0.05

[1] The exosome fraction was prepared by suspending the pellet after ultracentrifugation in the same volume of PBS as the original serum sample. Values are expressed as means ± SEM.

Table 2 : Body weight and concentration of total protein and adiponectin in serum and exosome fraction of +/+ and *ob/ob* mice

	+/+	***ob/ob***
Body weight (g)	28.5±2.0	48.7±2.3*
Serum		
Total protein (mg/ml)	51.6±0.4	64.2±1.4*
Adiponectin (ng/ml)	7,562±736	5,053±191*
Exosome fraction[1]		
Total protein (mg/ml)	0.43±0.07	0.67±0.04*
Adiponectin (ng/ml)	133±21	103±19

[1] The exosome fraction was prepared by suspending the pellet after ultracentrifugation in the same volume of PBS as the original serum sample. Values are expressed as means ± SEM. Comparison with mean values was done by student's T-test. * $P<0.05$ vs. +/+

Table 3 : Body weight and concentration of total protein and adiponectin in serum and exosome fraction of C57BL/6 mice fed normal- and high-fat diets

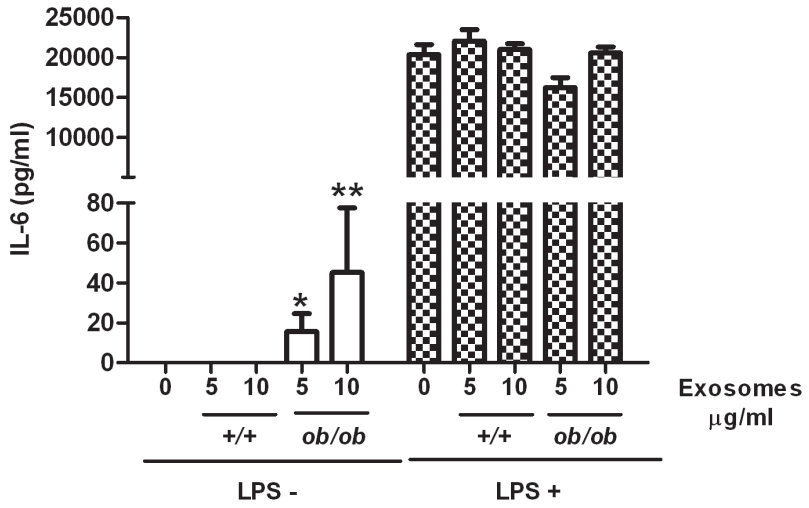
	Normal-fat diet	**High-fat diet**
Body weight (g)	27.8±0.8	51.4±0.5*
Serum		
Total protein (mg/ml)	43.4±1.2	45.6±1.5
Adiponectin (ng/ml)	5,403±439	4,497±336*
Exosome fraction[1]		
Total protein (mg/ml)	0.84±0.16	0.93±0.10
Adiponectin (ng/ml)	609±84	476±98

[1] The exosome fraction was prepared by suspending the pellet after ultracentrifugation in the same volume of PBS as the original serum sample. Values are expressed as means ± SEM. Comparison with mean values was done by student's T-test. * $P<0.05$ vs. +/+

3.4 Exosomes derived from obese mice induced macrophages activation *in vitro*

The concentration of IL-6 and TNF-α in the culture supernatant of RAW 264.7 cells was significantly higher in LPS-treated cells than untreated cells (Fig. 14a and b). In the cells without LPS treatment, IL-6 was undetected when exosomes isolated from +/+ mice were added. In contrast, IL-6 was increased by adding exosomes isolated from *ob/ob* mice in a dose-dependent manner ($P<0.05$) (Fig. 14a, left). However, no changes were observed in IL-6 concentration by adding exosomes isolated from +/+ and *ob/ob* mice under LPS-stimulated conditions (Fig. 14a, right). Although TNF-α was detected under LPS-untreated conditions, the concentration tended to be higher in the cells added by exosomes isolated from *ob/ob* mice than those added by exosomes isolated from +/+ mice (Fig. 14b, left). With LPS-stimulated conditions, no differences were observed between cells treated exosomes isolated from +/+ and *ob/ob* mice (Fig. 14b, right).

a.)



b.)

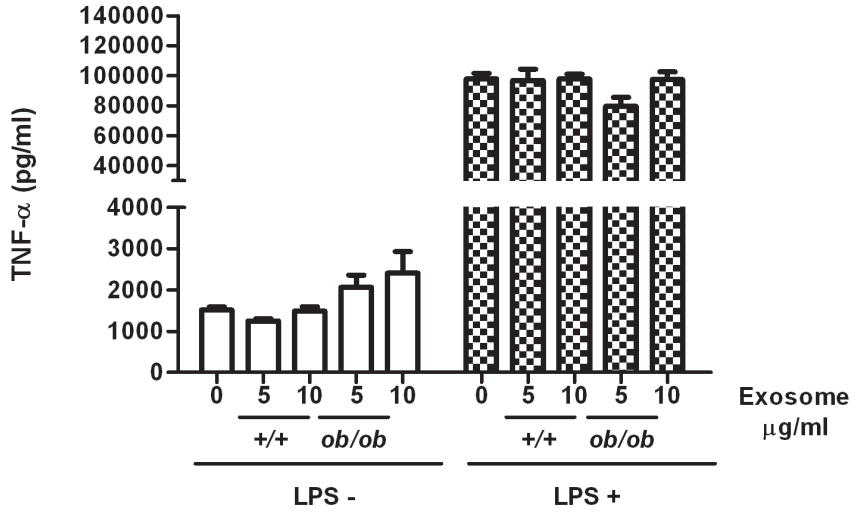


Fig. 14 : ELISA of a) IL-6 and b) TNF-α in RAW 264.7 macrophages supernatant after treated with 0, 5 or 10 μg/ml exosomes isolated from +/+ and *ob/ob* mice with or without LPS stimulation.

CHAPTER IV
DISCUSSION AND CONCLUSION

Based on western blotting, CD63, a well-known protein marker of exosomes (Vlassov et al., 2012), was concentrated in the pellet of mouse serum after ultracentrifugation, suggesting successful isolation of exosomes. In the exosome fraction, this study detected adiponectin and trace amounts of resistin, but not leptin. In addition, after ultracentrifugation on a discontinuous gradient, both adiponectin and CD63 were detected in a fraction at a density of 1.17 g/mL, consistent with the density of exosomes (Vlassov et al., 2012). Thus, it seems likely that a portion of the adiponectin is associated with exosomes in mouse serum. In fact, previous *in vitro* studies have demonstrated that mouse exosomes are associated with adiponectin. Deng et al. and Sano et al. respectively identified adiponectin by proteomic analysis in the exosomes released from cultured mouse adipose tissue and mouse adipocyte cell line 3T3-L1 (Deng et al., 2009; Sano et al., 2014). Given that adiponectin is produced exclusively by adipocytes, and considering that cultured adipose tissue and 3T3-L1 adipocytes release exosomes (Deng et al., 2009; Sano et al., 2014), it is possible that adiponectin-associated exosomes in serum are derived from adipocytes. In other words, adiponectin could be a marker for adipocyte-derived exosomes in the circulation.

Western blot analysis in this study showed that the signal intensity for CD63 in the exosome fraction was decreased by treatment with proteinase K, suggesting that CD63 is not an intrinsic protein but rather a transmembrane protein, which is consistent with previous reports (Vlassov et al., 2012). Along with CD63, adiponectin was sensitive to proteinase K treatment. A weak adiponectin signal remained after a 30 min treatment with proteinase K, whereas the combination of both proteinase K and Triton X-100 completely quenched the signal. These results suggest that adiponectin exists as a transmembrane protein in the serum exosomes and that the anti-adiponectin antibody this study used may recognize the transmembrane and extraexosomal domain of adiponectin. Thus, it may be possible to analyze and isolate adipocyte-derived exosomes by immunomagnetic beads using anti-adiponectin antibodies that recognize the extraexosomal domain of adiponectin.

Quantitative analysis by ELISA showed that the amount of adiponectin in the exosome fraction accounts for approximately 2–10% of total adiponectin in the serum. The physiological implications of partial association of serum adiponectin with exosomes remain to be clarified. Adiponectin exerts its physiological function via the transmembrane receptors AdipoR1 and AdipoR2 (Yamauchi & Kadowaki, 2013). AdipoR1 is a high-affinity receptor in the globular domain of adiponectin and is primarily expressed in skeletal muscle, whereas AdipoR2 has an intermediate affinity for the collagenous and globular domains of adiponectin and is abundant in the liver (Yamauchi & Kadowaki, 2013). Association of adiponectin with exosomes may influence the recognition of the collagenous, globular, or both domains of adiponectin by the receptors, thereby affecting its physiological function. Precise analysis of the structural aspects of adiponectin associated with exosomes would provide relevant information.

Serum concentration of adiponectin is reportedly decreased in obese human and rodent models (Arita et al., 1999; Hu, Liang, & Spiegelman, 1996). This study observed a lower concentration of serum adiponectin in *ob/ob* mice and HFD-fed obese mice when compared to lean mice. In addition, adiponectin knockout mice exhibit insulin resistance and diabetes (Kubota et al., 2002). Furthermore, administration of adiponectin reportedly lowers blood glucose levels, ameliorates insulin resistance (Berg et al., 2001; Yamauchi et al., 2001) and increases fatty acid oxidation (Yamauchi et al., 2002). Thus, adiponectin protects against metabolic syndrome. Adiponectin exists as LMW, MMW and HMW forms in the circulation (Waki et al., 2003; Pajvani et al., 2002), and HMW adiponectins have a more relevant role in insulin sensitivity and in protecting metabolic diseases (Lara-Castro et al., 2005; Pajvani et al., 2004; Waki et al., 2003). In the present study, Western blotting following SDS–PAGE under nonreducing and unheated conditions indicated that, although MMW adiponectins are the predominant form in the serum, HMW adiponectins are present principally in the exosome fraction. These findings suggest that exosome-associated adiponectin and exosome-free adiponectin may have different physiological and pathological functions *in vivo*. It would be of interest to test whether the administration of exosome-associated adiponectin and exosome-free adiponectin exert different effects, particularly in terms of insulin-sensitizing and anti-

inflammatory functions, on recipient animals.

Because exosomes seem to reflect the diverse functional and dysfunctional states of the releasing cells, adipocyte-derived exosomes in the circulation could be influenced qualitatively and quantitatively by obesity. In fact, the total amount of exosomal proteins in the serum is reportedly higher in *ob/ob* mice than in wild-type mice (Sano et al., 2014). Similarly, this study observed that the protein concentration in the exosome fraction was higher in *ob/ob* mice than in +/+ mice, although there were no differences between HFD-fed obese and NFD-fed lean mice. In addition, cultured adipose tissue of *ob/ob* mice and diet-induced obese mice released more exosomal proteins than wild-type lean mice (Deng et al., 2009). Thus, it appears likely that obesity increases the release of exosomal proteins from adipose tissue, although the mechanism behind this release and the physiological implications remain unknown. This study also observed that obesity decreased the ratio of adiponectin to total protein in the exosome fraction. Because adiponectin expression is reduced in obese adipocytes (Arita et al., 1999), it could be presumed that the lower concentration of adiponectin in serum exosomes may reflect reduced production of adiponectin in obese adipocytes.

During obesity, adipocytes secrete exosomes, which are absorbed by monocytes, and the monocytes further migrate into adipose tissues and differentiate into macrophages (Hajer et al., 2008; Holvoet, 2012). Meanwhile, TNF-α activates $CD4^{+}$Th 1 cells and $CD8^{+}$effector T cells, leading to proinflmmatory macrophage stimulation (Holvoet, 2012; Lafontan, 2014). This study found that RAW 264.7 macrophages treated with exosomes isolated from *ob/ob* mice led to higher production of IL-6 and TNF-α when compared to +/+ mice. These results suggest that exosomes derived from obese mice promote activation of macrophages. Previously, Deng et al. (2009) reported that injection of exosomes isolated from culture supernatant of visceral adipose tissues excited from *ob/ob* mice led to increase in IL-6 and TNF-α levels and induce insulin resistance in recipient mice (Deng et al., 2009). They showed that exosomes isolated from culture supernatant of *ob/ob* mice were taken up predominantly by CD11bF4/80^{+} monocytes in recipient mice, followed by increased expression of monocyte receptors ICAM-1, CD204, and MHCII. In addition, bone marrow precursor cells treated with exosomes derived from cultured adipose tissues of

ob/ob mice exhibited differentiation into macrophages and secreted high level of IL-6 and TNF-α *in vitro*, while this differentiation did not occur when treated with exosomes derived from cultured adipose tissues of wild-type B6 mice. Addition of conditioned medium of macrophages pretreated with exosomes derived from cultured adipose tissues of *ob/ob* mice to C2C12 cells resulted in lower levels of phosphorylated Akt and inhibition of basal and insulin-stimulated transport of glucose, indicating impaired insulin function in myoblasts by such exosomes. Furthermore, wild-type B6 and TLR4 knockout mice were injected intravenously with exosomes derived from cultured adipose tissues of *ob/ob* and lean mice. The results showed that injection of exosomes derived from adipose tissues of *ob/ob* mice developed glucose intolerance and insulin resistance in wild-type mice. Moreover, the level of glucose intolerance, insulin resistance, and serum IL-6 and TNF-α were much lower in TLR4 knockout recipient mice. Taken together, Deng et al. (2009) found that exosomes released from adipose tissues of *ob/ob* mice substantially enhance the development of insulin resistance and the induction of inflammatory cytokines in a TLR4-dependent manner. Thus, the findings in this study are consistent with observations by Deng et al. (2009).

In conclusion, the present study showed that serum adiponectin is partially associated with exosomes in mice. Considering that adiponectin is produced exclusively by adipocytes, adiponectin associated exosomes in serum could be derived from adipocytes. Further studies are needed to clarify the physiological implications for the partial association of serum adiponectin with exosomes. Nevertheless, this study proposes that adiponectin could be a marker for exosomes released from adipocytes *in vivo*.

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APPENDICES

APPENDIX A
NUTRITION INFORMATION IN ANIMAL DIETS

1. Nutrition information of laboratory chow diet

Table 1: Nutrition information of laboratory chow diet[1]

Nutrition information	Amount per 100 g
Energy, kcal/g	231
Crude protein, g	18.8
Crude fat, g	3.9
Crude ash, g	6.9
Crude fiber, g	6.6
Nitrogen-free extract, g	54.7
Water, g	9.2
Vitamins	
Retinol, IU	1,615
β-Carotene, mg	0.12
Cholecalciferol, IU	317
α-Tocopherol, mg	4.52
Menadione, mg	1.54
Thiamine, mg	1.09
Riboflavin, mg	0.89
Ascorbic acid, mg	4.91
Pyridoxine, mg	1.89
Cyanocobalamin, μg	2.6
Inositol, mg	0.97
Biotin, mg	0.03
Pantothenic acid, mg	2.51
Niacin, mg	10.17
Choline, g	0.18
Folic acid, mg	0.26
Minerals	
Calcium, g	0.98
Phosphorus, g	0.80
Magnesium, g	0.26
Sodium, g	0.29
Potassium, g	0.97
Iron, mg	23.98
Copper, mg	1.42
Zinc, mg	8.87
Cobalt, μg	8.0
Manganese, mg	10.12

[1] Translated from http://lt.nosan.co.jp/ahg/pdf/nn_labo_01.pdf (in Japanese)

2. Nutrition information and ingredient composition of normal fat diet and high fat diet

Table 2: Nutrition information and ingredient composition of normal fat diet[1] and high fat diet[2]

Nutrition information	**Normal fat diet**		**High fat diet**	
	% g	% kcal	% g	% kcal
Protein	19.2	20	26	20
Carbohydrate	67.3	70	26	20
Fat	4.3	10	35	60
Total		100		100
kcal/g	3.85		5.24	
Ingredients	g	kcal	g	kcal
Casein, 30 Mesh	200	800		
Casein, 80 Mesh			200	800
L-Cystine	3	12	3	12
Corn starch	315	1260	0	0
Maltodextrin 10	35	140	125	500
Sucrose	350	1400	68.8	275
Cellulose, BW200	50	0	50	0
Soybean oil	25	225	25	225
Lard	20	180	245	2205
Mineral mix S10026	10	0	10	0
DiCalcium phosphate	13	0	13	0
Calcium carbonate	5.5	0	5.5	0
Potassium citrate, 1 H2O	16.5	0	16.5	0
Vitamin mix V10001	10	40	10	40
Choline bitartrate	2	0	2	0
FD&C Yellow Dye #5	0.05	0		
FD&C Blue Dye #1			0.05	0
Total	**1055.05**	**4057**	**773.85**	**4057**

[1]Diet D12450B, 10% kcal fat, Research Diets, Inc., USA

[2]Diet D12942, 60% kcal fat, Research Diets, Inc., USA

APPENDIX B
SDS-PAGE AND WESTERN BLOT REAGENTS

1. SDS-PAGE reagents

1.1 Stock solution

1.1.1 Upper buffer : 100 ml

2-Amino-2-hydroxymethyl-1,3-propanediol (Tris (hydroxymethyl)aminomethane), (Final 0.5M)	6.06 g
10% (w/v) Sodium dodecyl sulfate (SDS) solution (Final 0.4%)	4 ml

Procedure

1.) Dissolve Tris in 80 ml of DW

2.) Adjust pH to 6.8 with conc. HCl (use Pasteur pipet)

3.) Add 4 ml of 10% SDS solution

4.) Fill up to 100 ml, store at RT

1.1.2 Lower gel buffer : 100 ml

2-Amino-2-hydroxymethyl-1,3-propanediol (Tris (hydroxymethyl)aminomethane) (Final 1.5M)	18.17 g
10% (w/v) SDS solution (Final 0.4%)	4 ml

Procedure

1.) Dissolve Tris in 80 ml of DW

2.) Adjust pH to 8.8 with conc. HCl (use Pasteur pipet)

3.) Add 4 ml of 10% SDS solution

4.) Fill up to 100 ml, store at RT

1.1.3 10x running buffer : 500 ml

2-Amino-2-hydroxymethyl-1,3-propanediol (Tris (hydroxymethyl)aminomethane)	15.15	g
Glycine	72	g
SDS	5	g

Procedure

1.) Dissolve Tris, glycine, and SDS in 450 ml of DW

2.) Fill up to 500 ml, store at RT

1.1.4 3x sample buffer : 10 ml

Upper gel buffer	3.9	ml
SDS	0.9	g
Glycerol	3	ml
β-mercaptoethanol	1.5	ml
Bromophenol blue	1	mg
DW	1.6	ml

Procedure

1.) Dissolve all chemicals in DW

2.) Store at 4°C

1.1.5 30% Acrylamide solution : 100 ml

Acrylamide	29	g
N,*N'*-Methylenebis(acrylamide)	1	g

Procedure

*** Acrylamide is neurotoxin, wear gloves when prepare***

1.) Dissolve acrylamide and bis in 70 ml DW

2.) Fill up to 100 ml. Store at 4°C

1.6 10% APS : 10 ml

Ammonium peroxodisulfate	1	g
DW	10	ml

Procedure

1.) Dissolve ammonium peroxodifate in DW

2.) Divide into small aliquot and Store at -20°C

1.2 Gel preparation solutions

$$\textbf{Acrylamide (ml)} = \frac{\textbf{\% gel x vol. needed (ml)}}{\textbf{\% Acrylamide}}$$

Vol. needed = gel size (lower/upper part)

= width x length x depth (cm)

e.g. = 8 cm x 7 cm x 0.1 cm

= ~ 5.6 ml /gel

1.2.1 Lower gel buffer : 15 ml (2 gels)

	% Gel	
	7.5	**12**
Lower gel buffer (ml)	3.75	3.75
30% Acrylamide (ml)	3.75	6
DW (ml)	7.5	5.25
10% APS (µl)	75	75
TEMED (µl) (*N,N,N',N'*-tetramethylethylenediamine)	7.5	7.5

Procedure :

1.) Add lower gel buffer, 30% acrylamide, DW and 10%APS

2.) Mix by inverting the tube

3.) Add TEMED

4.) Mix by inverting the tube and immediately pour the gel

5.) Overlay DW by using syringe

6.) Leave the gel to set at least 20 min.

1.2.2 Upper gel buffer : 4 ml (2 gels)

Upper gel buffer	1	ml
30% Acrylamide	0.6	ml
DW	2.4	ml
10% APS	12	µl
TEMED	4	µl

Procedure :

1.) Add upper gel buffer, 30% acrylamide, DW and 10%APS

2.) Mix by inverting the tube

3.) Add TEMED

4.) Mix by inverting the tube and immediately pour the gel

5.) Insert comb

6.) Leave the gel to set at least 1.30 hr or overnight. Cover with plastic wrap to prevent moisture loss

1.3 1xrunning buffer : 500 ml

10x running buffer	50	ml
DW	450	ml

1.4 Coomasie Briliant Blue (CBB) staining

Discard MeOH containing solution to the organic waste

1.4.1 Prestain solution : 50 ml

AcOH	5 ml
MeOH	20 ml
DW	25 ml

Procedure :

1.) Mix all reagents

2.) Soak the gel with shaking for 15 min

1.4.2 CBB solution

Procedure :

1.) Soak the gel with shaking for 30 min

2.) Return the solution to the original container

1.4.3 Destain solution : 150 ml

AcOH	15 ml
MeOH	37.5 ml
DW	97.5 ml

Procedure :

1.) Mix all reagents

2.) Soak the gel with shaking for 20 min for 3 times

1.4.4 Fix solution : 50 ml

AcOH	5 ml
DW	45 ml

Procedure :

1.) Mix all reagents

2.) Soak the gel with shaking until background is clear

2. Western blot solutions

2.1 Stock solutions

2.1.1 10x Transfer buffer : 500 ml

2-Amino-2-hydroxymethyl-1,3-propanediol	15.15 g
(Tris (hydroxymethyl)aminomethane) (Final 250 mM)	
Glycine (Final 1920 mM)	72 g

Procedure :

1.) Dissolve Tris and glycine in 450 ml of DW

2.) Fill up to 500 ml with DW. Store at RT

2.1.2 10x TBS : 500 ml

NaCl	43.83 g
2M Tris-HCl, pH 7.5	50 ml

Procedure :

1.) Dissolve NaCl and 2M Tris-HCl, pH 7.5 in 400 ml of DW

2.) Fill up to 500 ml with DW. Store at RT

2.1.3 10% Tween 20/PBS : 50 ml

Tween 20	5 g
(Polyoxyethylene (20) Sorbitan Monolaurate)	
PBS fill up to 50 ml	

2.2 Transfer buffer : 150 ml

	% Gel	
	7.5	**12**
10x Transfer buffer (ml)	105	75
MeOH (ml)	30	30
DW (ml)	15	45

2.3 0.9% NaCl : 50 ml

NaCl	0.45 g
DW	50 ml

2.4 Blocking buffer : 50 ml

BSA	0.25 g
10x TBS	5 ml
10% Tween 20/PBS	500 µl
DW fill up to 50 ml	

2.5 1xTBS-T : 300 ml

10x TBS	30 ml
10% Tween 20/PBS	3 ml
DW	267 ml

APPENDIX C

REAGENTS FOR DISCONTINUOUS IODIXANOL (OptiPrep™)-SUCROSE GRADIENT CENTRIFUGATION

1. 0.25 M Sucrose, 10 mM Tris-HCl, pH 7.4

For 50 ml :

Sucrose	4.28	g
Tris	250	µl
DW up to 50 ml		

2. Iodixanol (OptiPrep™)-sucrose solution for centrifugation

For 8 ml :

% Iodoxanol-sucrose solution	OptiPrep™ (ml)	0.25 M Sucrose , 10 mM Tris-HCl, pH 7.4 (ml)
6	0.8	7.2
12.5	1.67	6.33
25	3.33	4.67
50	6.67	1.33

Note : OptiPrep™ contains 60% Iodixanol

3. Iodixanol (OptiPrep™)-sucrose standard solution for density determination

For 150 µl :

% Iodoxanol-sucrose solution	OptiPrep™ (µl)	0.25 M Sucrose , 10 mM Tris-HCl, pH 7.4 (µl)
0 (Blank)	0	150
6	15	135
12	30	120
24	60	90
32	80	70
36	90	60
44	110	40
50	125	25

APPENDIX D

ABSORBANCE (340 nm) AND DENSITY OF IODIXANOL SOLUTION ON 0.25 M SUCROSE SOLUTION

% Iodixanol	$A_{340\ nm}$	Density (g/ml)	% Iodixanol	$A_{340\ nm}$	Density (g/ml)
6	0.112	1.059	30	0.550	1.175
8	0.148	1.069	32	0.537	1.185
10	0.184	1.079	34	0.625	1.194
12	0.220	1.088	36	0.662	1.204
14	0.256	1.098	38	0.698	1.214
16	0.294	1.107	40	0.735	1.223
18	0.330	1.117	42	0.772	1.233
20	0.366	1.127	44	0.808	1.243
22	0.403	1.136	46	0.845	1.252
24	0.440	1.146	48	0.882	1.262
26	0.477	1.156	50	0.918	1.272
28	0.514	1.165			