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# A comparative study between nanoparticle-targeted therapeutics and bioconjugates as obesity medication

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**Abstract:**

Antiangiogenesis has been the focus of a new strategy for the treatment of obesity. However, little is known regarding the issue of whether targeting angiogenesis by nanoparticle-targeted therapeutic is advantageous or not in debugging the co-morbidity associated with diet-induced obesity (DIO) and the metabolic syndrome. We report herein on the positive effect of prohibitin (an adipose vascular marker)-targeted nanoparticle (PTNP) encapsulated in a proapoptotic peptide [$_{D}$(KLAKLAK)$_2$, KLA] on DIO and dysfunctional adipose tissue, a major mediator of the metabolic syndrome, as evidenced by ectopic fat deposition. The systemic injection of DIO mice with a low dose of KLA-PTNP, rather than a bioconjugate composed of the same targeting peptide and KLA (Adipotide) resulted in a reduction in body weight, as evidenced by a significant decrease in serum leptin levels, in parallel with an antiobesity effect on dysfunctional adipose cells, including adipocytes and macrophages. In addition, the KLA-PTNP treatment resulted in a reduction in ectopic fat deposits in liver and muscle with the lipolytic action of elevated serum adiponectin, with no detectable hepatoxicity. Notably, drug delivery via PTNP that had accumulated in obese fat via the enhanced permeability and retention effect was enhanced by multivalent active targeting and cytoplasmic delivery into adipose endothelial cells via escaping from endosomes/lysosomes. Thus, vascular-targeted nanotherapy has the potential to contribute to the control of adipose function and ectopic fat deposition associated with obesity and the metabolic syndrome.

**Key Words:**

Nanoparticle-targeted proapoptotic peptide, Adipotide, Diet-induced obesity, metabolic syndrome, Ectopic fat, Antiangiogenesis

## 1. Introduction

Obesity and its-related metabolic syndrome is closely associated with the microenvironment associated with adipose tissue [1-3]. A major cause of obesity is the over-eating of energy-rich food, which, in turn, accumulates in the parenchymal adipocytes of adipose tissue, a major mediator of the metabolic syndrome that is manifested by ectopic fat deposition, particularly in liver and muscles [1-3]. The process, which is involved in the deposition of lipids as triglycerides (TG) into adipocytes, is known as adipogenesis which relies on the simultaneous development of angiogenesis [4-5]. In addition, endothelial cells play a critical role in the development of inflammation, including the adhesion and recruitment of leukocytes to inflammatory sites and the secretion of inflammatory cytokines and chemokines [5-6]. Therefore, it would be expected that suppressing angiogenesis would inhibit the progression of adipocyte hyperplasia and palliate insulin resistance by reducing the infiltration of inflammatory cells, including macrophages, into adipose tissue.

Regarding the targeting of adipose vessels with the goal of controlling obesity, Kolonin, MG *et al*. reported on a small peptide (KGGRAKD) that specifically binds to endothelial cell-surface prohibitin in white fat vessels (WFV) and subsequently fuses with a cell death-inducing peptide [$_{D}$(KLAKLAK)$_2$, KLA]. The *s.c* injection of the peptidomimetic CKGGRAKDC-GG-$_{D}$(KLAKLAK)$_2$, termed Adipotide, was reported to promote weight loss in obese animals [7-8]. Thus, the disruption of the adipose vasculature by antiangiogenic therapeutics is a promising strategy in terms of the inhibition/depletion of angiogenesis-dependent adipogenesis (obesity).

We previously reported that PEGylated nanoparticles passively accumulate in obese fat via the enhanced permeability and retention (EPR) effect, as has been well established in tumor targeting [9-10]. Thus, to target angiogenic vessels in obese fat using a dual-targeting strategy: passive accumulation and active recognition, we designed and prepared prohibitin (an adipose vascular marker)-targeted nanoparticle (PTNP) whose surface was modified with a linear peptide containing a WFV-targeting motif (KGGRAKD), attached via a long polyethylene glycol (PEG) spacer to reduce the steric hindrance associated with ligand-receptor interactions and a short PEG-polymer as a surface biostabilizer to accelerate plasma circulation time [9, 11-12]. Thus, incorporation of KLA into the PTNP system (KLA-PTNP) might be shown promise for weight loss in DIO mice [9]. However, the effect of the KLA-PTNP system on the co-morbidity of DIO and metabolic syndrome is still unknown.

Our goal of this study was to develop a better understanding of the advantageous effect of nanoparticle-targeted therapeutics over that achieved when a bioconjugate composed of the same targeting peptide and the proapoptotic peptide (KLA) (Adipotide) is used on the comorbidity associated with DIO and dysfunctional adipose tissue, a major mediator of the metabolic syndrome that is manifested by ectopic fat deposition in the liver and muscle. Compared to the use of Adipotide, treatment with KLA-PTNP caused a potential decrease in body weight in parallel with serum leptin levels, adipocyte size, macrophage content and adipogenic/angiogenic clusters, while ectopic fat deposited in liver and muscle was significantly decreased via the lipolytic action of elevated serum adiponectin, suggesting that vascular-targeted nanotherapy may be effective for the control of the adipose function and peripheral deposited fat in the body. In addition, drug delivery via the PTNP system that was accumulated in obese fat was mediated by its multivalent active targeting, resulting in a subsequent enhancement in drug delivery to adipose endothelial cells.

## 2. Materials and Methods

### 2.1 Animals and Reagents

Diet-induced obese (DIO) mice (male, C57Bl/6J, body weight ≥ 40 g) or male *wild type* C57BL/6J mice (6-wks) were purchased from the Charles River Laboratories Japan (Yokohama, Japan) and from SLC Japan (Shizuoka, Japan) respectively. The mice were kept in individual cages on a 12 h day/night cycle and fed a high-fat diet (HFD, 58Y1, 34.9% fat, 23.1% protein and 25.9% carbohydrates) and a normal diet (ND, EQ 5L37, 4.5% fat, 25.0% protein and 49.3% carbohydrates) that were purchased from the PMI Nutrition International (Richmond, IN, ISA). All animal experiments were approved by the research advisory committee of Hokkaido University, Sapporo, Japan and performed in accordance with the guidelines for the Care and Use of Laboratory Animals. All animals were acclimatized for one week prior to use.

$PEG_{5kDa}$-DSPE and $PEG_{2kDa}$-DSPE with a functional maleimide moiety at the terminal end of the PEG: N-[(3-maleimide-1-oxopropyl) aminopropyl polyethylene glycolcarbamyl] distearoyl-*sn*-Glycero-3-phosphoethanolamine was bought from Nippon Oil and Fat Co. (Tokyo, Japan). 1, 2-dioleolyl-*sn*glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhodamine-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Peptides (GKGGRAKDGGC, $[_{D}(KLAKLAK)_2$, KLA] and CKGGRAKDC-GG-$_{D}(KLAKLAK)_2$ were

synthesized by Toray Industries Inc. (Tokyo, Japan) and GKGGRAKDGGC-rhodamine was prepared by Scrum Inc. (Tokyo, Japan). All of these peptides were purified by high-performance liquid chromatography to >95% purity.

### 2.2 Nanoparticle preparation and characterization

Prohibitin-targeted nanoparticles (PTNP) comprised of egg york phosphatidylcholine (EPC) and cholesterol (Chol) modified with a prohibitin targeting peptide-PEG-lipid conjugate (Pep-$PEG_{5kDa}$-DSPE, 1.25 mol% of total lipids) and $PEG_{2kDa}$-DSPE (1 mol% of total lipids) were prepared by a previously described reverse phase evaporation (REV) method [10]. The sizes and zeta potentials of the KLA-PTNP that were determined by photon correlation spectroscopy on a Malvern Zetasizer (Malvern instruments, Malvern, UK), were 109.2 ± 7.8 nm and 6.0 ± 0.9 mV respectively. The recovery ratio of KLA and rhodamine was 9.4 ± 3.4 and 2.8 ± 0.5%, respectively [9].

### 2.3 Anti-obesity study

DIO mice were allowed access to a HFD because this strain is prone to developing obesity throughout their lifespan. After the body weight reached around ≥ 44g, the mice were randomly divided into 4 groups. Three groups of at least three mice per group were exposed to the HFD along with an intravenous injection of 0.2 mmol/kg of Empty-PTNP (E-PTNP), 1 mg/kg of KLA-PTNP and 3 mg/kg of Adipotide at 3 day intervals for 30 days whereas the other group (n=3) remained untreated and served as non-treated controls. Mouse body weight was measured every 3 days during therapy. After the treatment, the mice (four groups) were sacrificed and blood was withdrawn by cardiac puncture under anesthesia. The tubes that contained blood were allowed to stand at room temperature for 3h and serum was then separated by centrifugation for 10 min at 1400 × *g*. Whole serum portions were immediately stored at -20°C for measurement of blood parameters. Epididymal and subcutaneous fat pads were removed and images obtained using a digital camera.

### 2.4 Histopathology

Immunostaining of formalin-fixed obese adipose tissue pieces for the identification of $F4/80^+$ macrophages was performed as described in a previous report [6]. For the staining of formalin-

fixed tissue pieces with boron dipyrromethene (BODIPY), a FITC- or Alexa647-Griffonia simplicifolia isolectin (GSIB4) (Vector Lab, Burlingame, CA, USA) and nuclei with Hoechst33342 (Invitrogen, Carlsbad, CA, USA) were used, following a previously described procedure [5]. Slices (around 100 μm in thickness) of liver and muscle tissues were obtained using a microslicerTM DSK-1000 (Dosaka EM. Co. Ltd., Kyoto, Japan) and the resulting slices were then stained with BODIPY, rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA, USA) and Hoechst 33342. After mounting the specimens on glass slides, they were examined by confocal microscopy (CLSM) (Nikon, A1). For the quantification of fluorescence of BODIPY, rhodamine-phalloidin and Hoechst33342, we used Image-Pro(R) Plus-4.5 software (Media Cybernetics, Inc. Rockville, USA).

**2.5 Determination of adipocyte size**

We determined adipocyte size as described in a previous report [5]. Briefly, five low-power field images were acquired at regular spatial intervals from an epididymal fat pad of each mouse in a group (3 mice per group), after which the diameters of 20 cells in each field were measured by an observer who was blind to the conditions. Adipocytes were defined by regularly round $BODIPY^+$ cells without a disruption in the plasma membrane.

**2.6 Measurement of blood parameters**

The serum samples from the four groups were defrosted at room temperature and analyzed. Serum leptin and adiponectin levels were determined by ELISA assay (R&D Systems, Inc. Minneapolis, MN). Serum ALT was determined using a commercially available kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). These assays were performed according to the manufacturer's suggested protocols.

**2.7 Confocal observation of living tissues**

Mice (DIO C57BL/6J mice (>42g)) were intravenously administered the rhodamine-loaded PTNP or rhodamine-labeled ligand at a rhodamine dose of 0.2 mmol/kg. The Recovery Ratio of rhodamine (2.96 ± 0.5%) encapsulated in PTNP was taken into account in the dose calculation. To visualize blood vessels, FITC-IB4 was injected intravenously (50 μg/mice) 30 min prior to tissue collection. The mice were anesthetized and as much blood as possible removed by cardiac

puncture. Tissues from the adipose epididymal region were collected and washed 3 times with Hank's Buffered Salt Solution (HBSS) and then cut into small pieces using a sterile technique, as described previously [9]. After washing with HBSS, the pieces were transferred to light-protected disposable tubes containing HBSS and placed on ice until used. The pieces that were transferred to glass-base dishes were viewed under a CLSM.

### 2.8 Cellular event studies

Primary endothelial cells from inguinal adipose tissue (pcEC-IWAT) were isolated as described previously [13]. For the quantitative measurement of cellular uptake, pcEC-IWAT cells ($5 \times 10^4$) were seeded in a 24-well plate, pre-coated with 1.5% gelatin (SIGMA-Aldrich, Tokyo, Japan) and 10 μg/ml human fibronectin (Asahi Glass, Tokyo, Japan), in the presence of EGM-2MV media (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, CA, USA) and 0.1 mg/ml kanamicin sulfate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) overnight at 37◦C in an atmosphere of 5% $CO_2$ and 100% humidity. Before incubating the rhodamine-loaded PTNP and rhodamine-labeled ligand with the pcEC-IWAT cells, the initial fluorescent intensity of rhodamine was adjusted and the same rhodamine doses (0.2 μmol/l) were added. The sample was then incubated for an additional 1 h under the same conditions. After the incubation, the cells were washed 3 times with 1ml of heparin in PBS (40 units/ml) and then treated with Reporter Lysis Buffer (Promega Corp., Madison, WI, USA) followed by centrifugation at 12,000 rpm for 5 min at 4◦C to remove debris. The efficiency of cellular uptake of the rhodamine-loaded PTNP and rhodamine-labeled ligand were determined by measuring the fluorescence intensity of rhodamine (excitation at 550 nm and emission at 590 nm) using a FP-750 Spectro-fluorometer (JAS Co, Japan). For the endosomal escape study, pcEC-IWAT cells were prepared as described previously [12] and incubated with the rhodamine-loaded PTNP and rhodamine-labeled ligand at the same dose for 3 h, followed by washing with heparin in PBS, stained with lysosensor green DND-189 and were observed by CLSM.

### 2.9 Statistical analysis

All statistical analyses were performed using the JMP6 statistical package (SAS Institute, Cary, NC). One-way ANOVA followed by Dunnett's multiple comparison and Tukey-Kramer's HSD test was used to evaluate statistical significance. The paired *t*-test was also used to determine the

statistical significance of each treatment on body weight. A P value of $< 0.05$ was considered to be significant.

## 3. Results

### 3.1 Enhanced antiobesity activity of the nanoparticle-targeted proapoptotic peptide

To investigate the effect of targeted-KLA for weight loss, we prepared the KLA-PTNP system and Adipotide, as illustrated in **Figure 1A**. We then treated DIO mice that had been fed a high-fat diet (HFD) with these preparations at 3 day intervals for a total of 30 days by i.v-injections of KLA-PTNP and Adipotide at doses of 1.0 and 3.0 mg/kg, respectively. Weight gain for the non-treated (NT) and empty-PTNP treated (E-PTNP) mice was accelerated by HFD feeding, whereas weight gain for both the Adipotide and KLA-PTNP mice was significantly less than that of NT group (**Figure 1B**). On day 30 of the treatment period, the body weights of the KLA-PTNP treated mice were significantly less than their initial weight (a 14% reduction), whereas that of the Adipotide treated mice was not (a 5% reduction) (**Table 1**). After the treatment, the size and length of excised subcutaneous and epididymal adipose tissues from the KLA-PTNP mice were decreased substantially, compared to the other groups (**Figure 1C**). Notably, the KLA-PTNP treatment also resulted in a substantial reduction in adipocyte size, compared to NT and E-PTNP, whereas a negligible decrease in adipocyte size was found in the case of Adipotide (**Figure 1D**). Overall, these results provide strong evidence to indicate that mice treated with KLA-PTNP lost a substantial amount of body weight as the result of a decrease in adipocyte tissue and adipocyte hypertrophy.

### 3.2 Effect of the KLA-PTNP treatment on dysfunctional adipose tissue

We next examined the histology of adipose tissue from the treated mice. Treatment with KLA-PTNP reduced the number of adipogenic/angiogenic clusters, compared to the NT, E-PTNP and Adipotide (**Figure 2A**), demonstrating that KLA-PTNP appears to have the ability to cause the regression of angiogenesis-dependent adipogenesis. Moreover, it is known that macrophages participate in aggravating the formation of clusters in DIO [6]. The results of an immunohistological analysis clearly showed that the KLA-PTNP treatment resulted in the elimination of F4/80+ macrophages from these clusters, resulting in a total decrease in the overall macrophage content in adipose tissue (**Figure 2B**).

### 3.3 Reversal of deposited ectopic fats in liver and muscle

To determine the nature of the beneficial and/or any toxic effects related to this treatment, we examined liver and muscle, both of which are closely associated with obesity and its related metabolic syndrome [1-3, 16-17]. A histological evaluation showed high levels of fat deposits in the liver and muscle tissues of obese mice, whereas the tissues from KLA-PTNP mice had regained their normal histological appearance with reduced fat accumulation (**Figure 3A**). An image analysis showed an approximately 58- and 13-fold decrease in accumulated ectopic fat in the liver and skeletal muscles of KLA-PTNP mice, compared to the NT controls (**Figure 3B and C**). The Adipotide treatment also resulted in a significant reduction in the accumulation of ectopic fat in liver and muscle, however, the therapeutic efficacy of KLA-PTNP was considerably higher than that of Adipotide.

### 3.4 Effect of the KLA-PTNP treatment on obesity-related biomarkers and toxicities

We also measured the serum levels of adipokines, leptin and adiponectin, which are known obesity-related biomarkers. Treatment of mice with Adipotide showed a marginal effect on serum leptin and adiponectin levels, compared to NT mice whereas a significant reduction and elevation in serum leptin and adiponectin levels, respectively, were found in the KLA-PTNP mice compared to NT mice (**Figure 4A and B**). It has been reported that serum leptin levels are correlated with the percentage of body fat [14] whereas adiponectin levels are reduced in obese rodents and humans [15]. Therefore, the reduction in leptin and the elevation adiponectin levels in serum after the KLA-PTNP treatment can be attributed to the reversal of obesity. In addition, the serum levels of liver enzymes, alanine aminotransferase (ALT) an elevation in serves as an indicator of hepatotoxicity, had a tendency to decrease as a result of this treatment (not statistically significant) (**Figure 4C**).

### 3.5 PTNP enhanced the drug delivery into adipose endothelial cells via a dual-targeting mechanism: passive accumulation and multivalent active targeting

To explore the cause for the greater effectiveness of KLA-PTNP vis-à-vis Adipotide in obesity therapy, we compared the cellular movement of the fluorescent-labeled drug-carrier (ligand and PTNP) to adipose endothelial cells. When DIO mice were intravenously injected with rhodamine-PTNP, the carrier accumulated in obese fat at high levels, compared to that of the ligand peptide alone (**Figure 5A**). These results suggest that a nanosized carrier may facilitate the passive accumulation of a drug into obese fat which is consistent with our recent finding [9] and this may enhance the subsequent binding of multiple ligands to its multiple receptors. In addition, to evaluate whether multivalent PTNP enhances cellular uptake, we treated primary adipose endothelial cells (pcEC-IWAT) with both rhodamine-loaded PTNP and rhodamine-labeled ligand at a fluorescent dose (0.2 μmol/l). The PTNP system showed approximately a 5-fold higher fluorescent uptake, compared to that of the rhodamine-labeled ligand. These results indicate that the PTNP system, owing to its multivalent potential, was strongly associated with its target on the cell surface, thereby promoting active targeting (**Figure 5B)**. We next examined the intracellularly delivered cargo marker. The incubation of pcEC-IWAT cells with rhodamine-loaded PTNP resulted in the intracellular appearance of rhodamine fluorescence, indicating that the internal cargo marker had successfully escaped from endosomes/lysosomes and was efficiently delivered to the cell cytoplasm, compared to that of cargo-carried by the ligand (**Figure 5C**).

## 4. Discussion

It is now generally accepted that antiangiogenesis represents a promising strategy for the control of obesity. Emerging evidence indicates that treatment with antiangiogenic drugs (TNP 470, curcumin, matrix metalloproteinase inhibitors) result in a reduction in weight gain in diet-induced obese (DIO) animals through the inhibition and /or destruction of adipose angiogenic vessels [18-20]. In the present study, we investigate whether the use of a proapoptotic peptide-loaded in a prohibitin-targeted nanoparticle (KLA-PTNP) would be effective or not in reducing angiogenesis-dependent adipogenesis in DIO and dysfunctional adipose tissue, a major mediator of the metabolic syndrome that is manifested by ectopic fat deposition in the liver and muscle, compared to that of a bioconjugate (Adipotide).

Adipose tissue is mainly composed of parenchymal adipocytes, endothelial cells and macrophages. The growth of adipose tissue (adipogenesis) is dependent on the concomitant

development of angiogenesis [4-5]. Therefore, neovascularization coupled to the formation of new adipocytes (the formation of adipogenic/angiogenic clusters) was accelerated in obese adipose tissue which is one of the key features of DIO [5]. It has also been reported that stromal cells (macrophages, immune cells) play a vital role in stimulating the formation of these clusters [6] and the vascular permeability in obese fat ultimately becomes increased as a hallmark of inflammation [6]. Taken together, the obese microenvironment might be sufficiently similar to exhibit the enhanced permeability and retention (EPR) effect which is well-known in tumors.

We previously reported that non-targeted nanoparticles (NTNPs) such as PEGylated nanoparticles, when they are administered to obese mice, physically accumulate and extravasate in adipose tissue [9]. Therefore, an enhanced accumulation of the PTNP system was found in adipose endothelial cells of obese fat, which is thought to be mediated by a dual-targeting mechanism, namely, passive accumulation and active targeting. Thus, treatment of the PTNP system with a proapoptotic peptide (KLA) (KLA-PTNP) has the potential to reduce the weight gain in DIO mice through the destruction of adipose vessels [9]. In this study, we also found that treatment with KLA-PTNP promoted weight loss in DIO mice where empty-PTNP (E-PTNP) was also utilized as a placebo to obtain the net effect of KLA (**Figure 1B**). However, the weight gain for KLA-PTNP treated mice with a 3-fold lower dose was around 3-fold lower than that when Adipotide, a conventional bioconjugate system, was used (**Table 1**). In addition, we also found that treatment with KLA-PTNP was not associated with the reduction of food intake (data not shown). However, the question of whether vascular disruption as the result of treatment with KLA-PTNP has the ability to reduce fat gain needs to be addressed. As reported previously [18-21], the hypertrophy of adipose tissue in DIO is highly correlated with adipocyte hypertrophy. Histological analyses of adipose tissue revealed that the size of adipocytes in the case of the KLA-PTNP treatment group, compared to that of other groups was significantly decreased (**Figure 1C**). Thus, it might be assumed that KLA-PTNP induced adipose loss in the following ways. First, it destroys blood vessels via the KLA-mediated apoptosis of adipose endothelial cells. Therefore, the lipids that were flowing in the circulation after the intake of high energy-rich food did not accumulate in adipocytes. Thus, adipocyte hypertrophy was inhibited, which leads to the prevention of an increase in fat mass. Second, the destruction of blood vessels in adipose tissue leads to the decrease in the supply of basic nutrients such as glucose. Therefore, adipocytes might use their own fat as energy source for their survival. This causes a decrease in

the size of adipocytes. Third, the destruction of blood vessels also decreases the supply of O2, assuming that the death of adipocytes could occur. Adipocyte hypotrophy and death lead to the loss of fat mass in the body. It is also known that the degree of adiposity is directly proportional to the content of infiltrated macrophages [22]. After a 30 day treatment with the KLA-PTNP system, the numbers of adipogenic/angiogenic cell clusters and infiltrated macrophages had regressed substantially in adipose tissue (**Figures 2A and 2B**). Thus, these findings provide evidence to show that the destruction of adipose endothelial cells by nanoparticle-targeted therapeutics may potentially have an effect on dysfunctional adipose cells, including adipocytes and macrophages, thereby contributing to the amelioration of adipose tissue microenvironment.

Dysfunctional adipose tissue of DIO is highly associated with the accumulation of fat in ectopic sites, particularly the liver and muscle tissue, which are major players in the obesity-related metabolic syndrome [1-3]. To investigate whether the KLA-PTNP treatment may be advantageous, in terms of ectopic fat loss, we analyzed the fat content in liver and muscle tissue. The results indicate that the KLA-PTNP treatment resulted in an approximately 58- and 13-fold decrease in accumulated ectopic fat in the liver and muscle respectively (**Figures 3A, B and C)**. It is likely that, because PTNP is tightly bound to prohibitin, which is expressed at relatively high levels on the surface of endothelial cells of adipose vessels and delivers its payload into the cytosol via prohibitin-mediated endocytosis [9, 12], it does not enable the delivery of KLA in the liver vessels. This scenario assumes that the loss of adipose vessels inhibits the supply of energy source such as free fatty acid from adipocytes to the other organs including liver and muscle. Therefore, liver and muscle cells might expend their own fat as energy source that results in the reduction of peripheral fat contents. Therefore, these data demonstrate that the reduction in ectopic fat content in liver and muscles as the result of the KLA-PTNP treatment may be very useful for preventing the pathology associated with the obesity-related metabolic syndrome.

DIO is involved in the up- and down-regulation of bio-macromolecules that facilitate the development of obesity, its-related metabolic syndrome and associated toxicities [23, 24]. Leptin has been shown to stimulate angiogenesis through the control of adipogenesis in DIO [24], whereas adiponectin assists in the stabilization of angiogenic vessels and the oxidation of fat in liver and muscle tissue [25]. Treating the DIO mice with KLA-PTNP potentially resulted in reduced serum leptin levels (**Figure 4A)**, suggesting that the decrease in weight, size of fat depots and adipocyte is consistent with the above scenario. An elevation in serum adiponectin

levels was also observed (**Figure 4B)**, demonstrating that the reduction in fat in liver and muscle is mediated by the adiponectin-induced oxidation of fat. It thus appears that KLA-PTNP has the ability to correct for the obesity-related biomarkers through the up- and down-regulation of serum adipokines. In addition, the evaluation in serum levels of liver enzymes, such as ALT in the KLA-treated mice using an ALT assay, indicated that no detectable hepatotoxicity develops as a result of the KLA-PTNP treatment on day 30 (**Figure 4C)**. As described above, KLA-PTNP showed greater beneficial pharmacological effects at a lower dosage compared to Adipotide, although both contain the same targeting and proapoptotic peptide. In the pharmacological examinations, the less effects of Adipotide might be resulted from a difference in the route of administration. Kolonin et al. used subcutaneous route whereas intravenous route is herein utilized for the comparison between KLA-PTNP and Adipotide.

To investigate the cause for the higher potential of KLA-PTNP vis-à-vis Adipotide in the treatment of obesity, we compared the accumulation of the fluorescent-labeled drug-carrier (ligand and PTNP) in adipose tissue. As expected, the accumulation of PTNP in obese fat was enhanced by passive accumulation through the EPR-like mechanism, whereas the peptide ligand mediated only active accumulation (**Figure 5A**). These results suggest that nanosized carriers may facilitate the accumulation of PTNP into obese fat. However, to absolutely verify this, it is necessary to evaluate the uptake of the PTNP system by adipose endothelial cells that had accumulated in adipose tissue. In addition, the PTNP system was taken up very efficiently by primary adipose endothelial cells (pcEC-IWAT), compared to the ligand peptide only (**Figure 5B**). It has been reported that nanoparticle-based multivalent interactions, the binding between multiple ligands and multiple receptors, promoted the cellular uptake of the carriers via multivalent enhancement of affinity to the target cells, compared to monovalent interactions [26, 27]. Therefore, the enhanced internalization of the PTNP system in adipose-derived endothelial cells might be mediated by multivalent interactions between multiple targeting ligands and multiple prohibitin receptors. However, in order to fully elucidate this possibility, further examinations such as determination of binding affinity of the PTNP system and ligand itself to adipose endothelial cells still remain to be clarified. Furthermore, an enhanced intracellular delivery of an internal cargo marker via the PTNP system, compared to that of the ligand peptide that had escaped from endosomes/lysosomes was found, indicating that the PTNP system might have the potential to deliver its loaded-therapeutic cargo into the cytosol of adipose endothelial

cells (**Figure 5C**). However, details of the intracellular trafficking of both carriers remain to be clarified. Collectively, these data clearly demonstrate that the higher therapeutic effects of targeted nanotherapy are mediated by a dual-targeting process, namely, the passive accumulation and multivalent targeting activity of the PTNP system to adipose endothelial cells and enhanced drug delivery and escape from endosomes, resulting in potential weight loss through KLA-mediated apoptosis in adipose vascular cells.

In short, there are three advantageous effects of using the KLA-PTNP system over the bioconjugate system (Adipotide) in the treatment of DIO. The first is that the KLA-PTNP system with a 3-fold lower dose should exert a higher therapeutic potential for weight loss. This is mediated by a dual-targeting mechanism: passive accumulation and multivalent active targeting. The accumulation of the PTNP system is boosted by passive targeting, while the multivalent targeting activity of the PTNP system enhances cellular uptake and the efficacy of the subsequent drug delivery involves escape from endosomes/lysosomes. The KLA-mediated apoptosis of adipose endothelial cells could potentially exert an antiobesity effect on parenchymal adipocytes and macrophages, thereby contributing to the amelioration of the adipose tissue microenvironment. The second is that the KLA-PTNP system has the potential to reduce ectopic fat content in liver and muscle tissue through the adiponectin-induced oxidation of fat. The third is that PTNP serves as a microreservoir, consisting of multi-KLA moieties, which allows them to be delivered in a controlled-release manner over a long period of time, whereas the ligand delivers KLA in a 1:1 ratio. A schematic illustration of this finding in a simplified form is shown in **Figure 6**.

## 5. Conclusion

In summary, the findings presented herein show that targeting angiogenic vessels by KLA-PTNP have the potential to be effective in terms of reducing weight gain via the control of adipose function. This effect may be advantageous for reversing ectopic fat storage in the liver and muscle of DIO mice, compared to the use of a chimeric peptide. The potential activity of PTNP might be mediated, not only by its multivalent active targeting, but also it results in an enhanced drug delivery to the cytosol by virtue of its ease of escaping from endosomes/lysosomes. It may therefore be feasible to deliver low molecular-weight angiogenesis inhibitors or therapeutic genes using the PTNP system to angiogenic vessels in obese fat to

control the abnormalities associated with adipose cells in DIO and its-related metabolic syndrome.

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**Figure Captions**

**Figure 1. Enhanced anti-obesity activity of nanoparticle-targeted proapoptotic peptide**. (**A**) Schematic illustration of a prohibitin-targeted nanoparticle (PTNP) encapsulating a proapoptotic peptide and Adipotide. (**B**) Body weight change in response to treatment. Diet-induced obese mice (n=3) exposed to a high fat diet were intravenously injected with KLA-PTNP (1 mg/kg), Adipotide (3 mg/kg), empty-PTNP (E-PTNP) or non-treated (NT) at 3 day intervals for 30 days. Mouse body weight was measured at 3 day intervals before injection. (**C**) The appearance of representative treated and control mice and their subcutaneous (SAT) and epididymal (EAT) adipose tissues at the end of the treatment period. Bars = 1 cm. (**D-E**) Formaldehyde-fixed epididymal adipose tissue from control C57BL/6J mice fed by normal diet and four groups of DIO mice (NT, E-PTNP, Adipotide and KLA-PTNP) were stained with BODIPY and observed by CLSM. Bars represent 100 μm (D). Average diameters of adipocytes. Adipocyte diameters were determined from normal control and four groups of DIO mice and the mean diameters are shown in the column graph (n=300 cells from three animals in each group) (E). Statistical analyses were performed with one-way ANOVA followed by Dunnett's multiple comparison test in (B) and Tukey-Kramer's HSD test in (E). Data represents the mean ± SD; ‡$P$<0.0005, †$P$<0.005 and *$P$<0.05.

**Figure 2. Effect of the KLA-PTNP treatment on dysfunctional adipose tissue.** (**A**) Adipogenic/angiogenic cell clusters, as visualized by tissue imaging. After treatment, EAT pieces were stained with BODIPY (blue), Alexa647-GSIB4 (red) and Hoechst33342 (green). The white arrow heads indicate clusters. Scale bars represent 100 μm. (**B**) Immunodetection of macrophages. Formalin-fixed EAT pieces were stained with antibody against F4/80 (red). Fat droplets (blue) and nuclei (green) were counterstained as described above. Bars = 50 μm.

**Figure 3. Reversal of ectopic fat deposition in liver and muscle.** (**A**) Fat accumulation in liver and skeletal muscle. Formalin-fixed liver and muscle specimens were stained with BODIPY (green), rhodamine-phalloidin (red) and Hoechst33342 (cyan). Scale bars represent 100 μm. (**B** and **C**) Quantification of fat in liver and muscle. Column graph indicates relative fat accumulation in liver (B) and muscle (C) where the green fluorescence of BODIPY in both

tissues was normalized to the cyan fluorescence of Hoechst33342 and red fluorescence of rhodamine-phalloidin respectively (n=3). Data are mean ± SD; ‡$P$<0.0005, †$P$<0.005 (one-way ANOVA followed by Tukey-Kramer's HSD test).

**Figure 4. Effect of the KLA-PTNP treatment on obesity-related biomarkers and toxicities.** (**A** and **B**) Serum leptin and adiponectin levels. Serum leptin (A) and adiponectin (B) levels after a 30 day treatment were determined by ELISA. (**C**) Serum ALT levels. The ALT values in serum from four groups were measured (n=3). Data represents mean ± SD; †$P$<0.005, *$P$< 0.05 (vs. NT control, one-way ANOVA followed by Dunnett's test).

**Figure 5. Enhanced drug delivery into adipose endothelial cells through multivalent PTNP that ware passively accumulated in obese fat.** (**A**) *In vivo* accumulation of ligand peptide and prohibitin-targeted nanoparticle into obese fat. DIO mice were *i.v*-injected with rhodamine-conjugated ligand peptide and rhodamine-loaded PTNP (red) at the same dose of rhodamine (0.2 μmol/kg). At 24h after injection, EAT pieces were stained with Alexa647-GSIB4 (green). Scale bars represent 100 μm. (**B-C**) Uptake and intracellular events of PTNP and ligand by primary adipose endothelial cells *in vitro*. Both rhodamine-PTNP and rhodamine-ligand (red) at a rhodamine dose of 0.2 μmol/l were incubated for 1 h (B). The relative cellular uptake is shown, where the rhodamine dose was normalized by the initial fluorescent intensity. Data are mean ± SD; n=3; #$P$<0.001 (vs. ligand, Student *t*-test). (C) 3 h. Endosomes were also stained with lysosensor green. Scale bar; 10 μm.

**Figure 6. Regulation of adipose function and deposited ectopic fat by vascular-targeted nanotherapy in diet-induced obesity.** Intravenously administered KLA-PTNP accumulates in obese fat through the enhanced permeability and retention effect and the multivalent active recognition and uptake of the PTNP system by adipose endothelial cells may occur via ligand-receptor interactions, thus releasing the drug into cytosol. The drug-induced apoptosis of endothelial cells occurs and this effect then affects adipocytes and macrophages that are associated with chronic inflammation. As a result, leptin levels are decreased and an adiponectin levels are increased in the blood circulation. The anti-inflammatory and elevated serum adiponectin levels assist in reducing the ectopic fat content in liver and muscle tissue.

**Table 1. Average body weight of mice before and after treatment.**

| Group | Body weight (g) | | *p*-value of paired *t*-test |
|---|---|---|---|
| | Before | After | |
| NT | 43.2 ± 8.1 | 50.2 ± 4.5 | 0.0828 |
| E-PTNP | 43.3 ± 5.3 | 46.2 ± 5.2 | 0.0024 |
| Adipotide | 43.8 ± 5.2 | 41.6 ± 3.8 | 0.2202 |
| KLA-PTNP | 44.5 ± 2.7 | 38.4 ± 3.9 | 0.0438 |

Data are the mean ± s.d. of 3 mice in each group. The paired *t*-test was performed to determine the differences between before and after treatment.

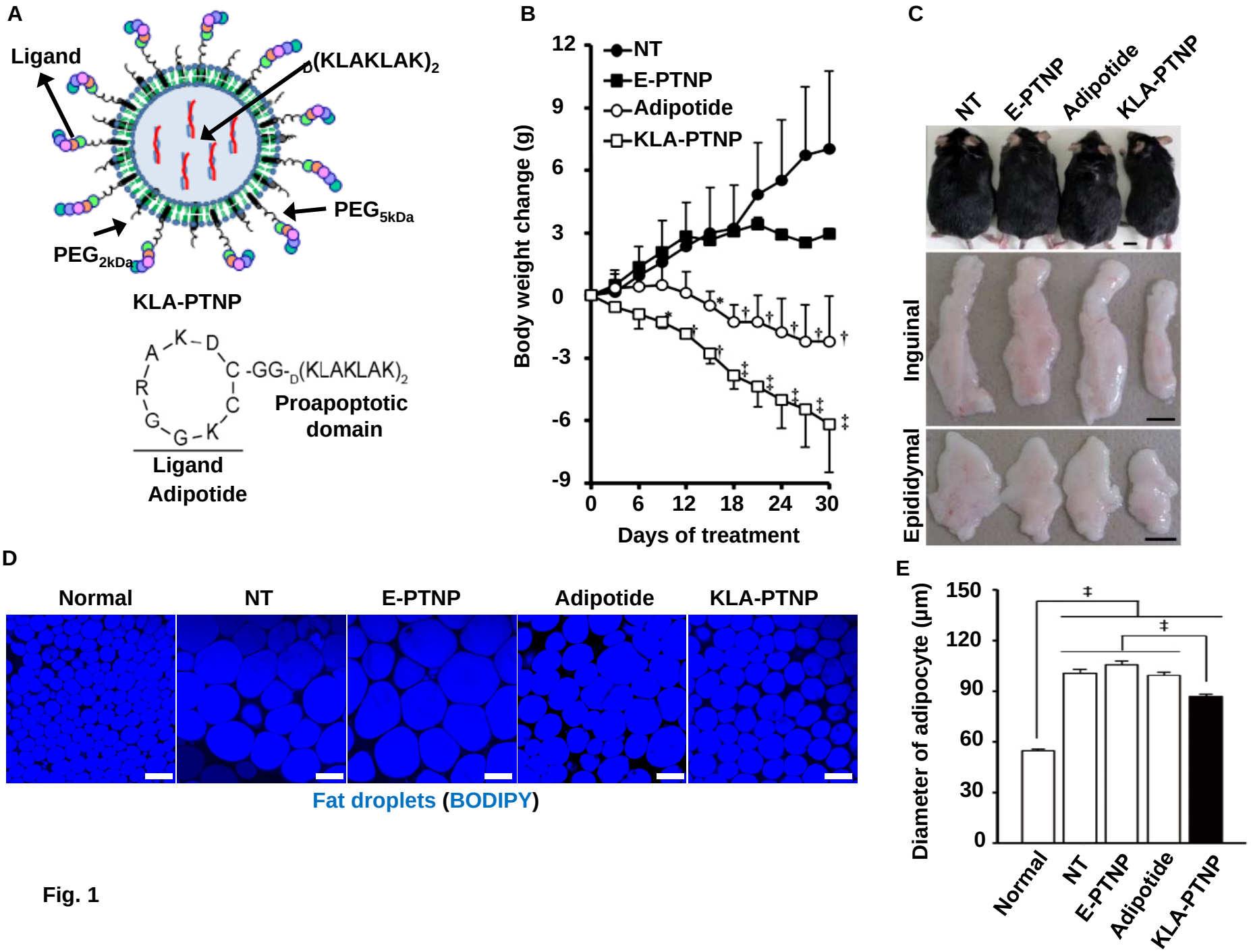
A
Ligand
D(KLAKLAK)2
PEG5kDa
PEG2kDa
KLA-PTNP
A K D C -GG-D(KLAKLAK)2
R C
G G K
Proapoptotic domain
Ligand
Adipotide
B
NT
E-PTNP
Adipotide
KLA-PTNP
Body weight change (g)
12
9
6
3
0
-3
-6
-9
0 6 12 18 24 30
Days of treatment
C
NT
E-PTNP
Adipotide
KLA-PTNP
Inguinal
Epididymal
D
Normal
NT
E-PTNP
Adipotide
KLA-PTNP
Fat droplets (BODIPY)
E
Diameter of adipocyte (μm)
150
120
90
60
30
0
Normal
NT
E-PTNP
Adipotide
KLA-PTNP

Fig. 1

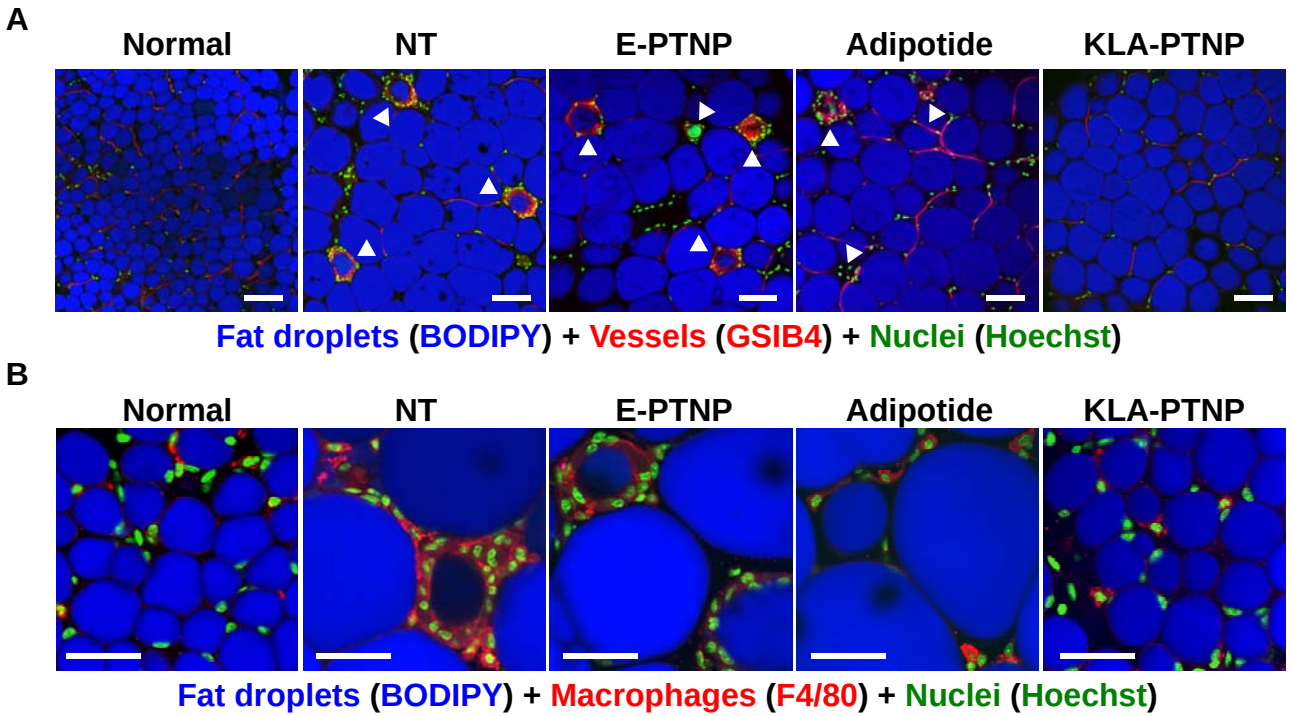
A
Normal
NT
E-PTNP
Adipotide
KLA-PTNP
Fat droplets (BODIPY) + Vessels (GSIB4) + Nuclei (Hoechst)
B
Normal
NT
E-PTNP
Adipotide
KLA-PTNP
Fat droplets (BODIPY) + Macrophages (F4/80) + Nuclei (Hoechst)

**Fig. 2**

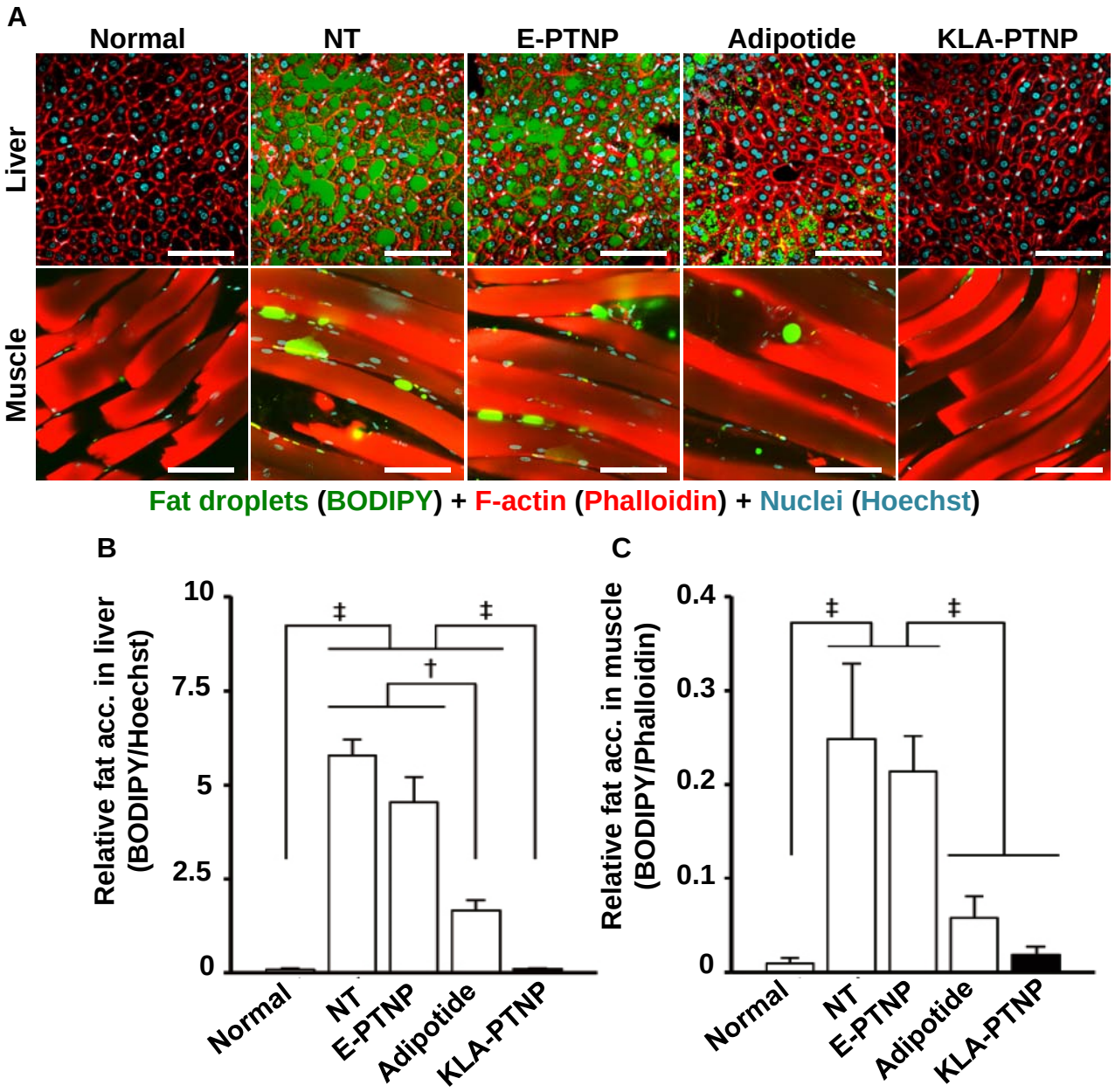
A
Normal
NT
E-PTNP
Adipotide
KLA-PTNP
Liver
Muscle
Fat droplets (BODIPY) + F-actin (Phalloidin) + Nuclei (Hoechst)
B
Relative fat acc. in liver (BODIPY/Hoechst)
10
7.5
5
2.5
0
‡
‡
†
Normal
NT
E-PTNP
Adipotide
KLA-PTNP
C
Relative fat acc. in muscle (BODIPY/Phalloidin)
0.4
0.3
0.2
0.1
0
‡
‡
Normal
NT
E-PTNP
Adipotide
KLA-PTNP

Fig. 3

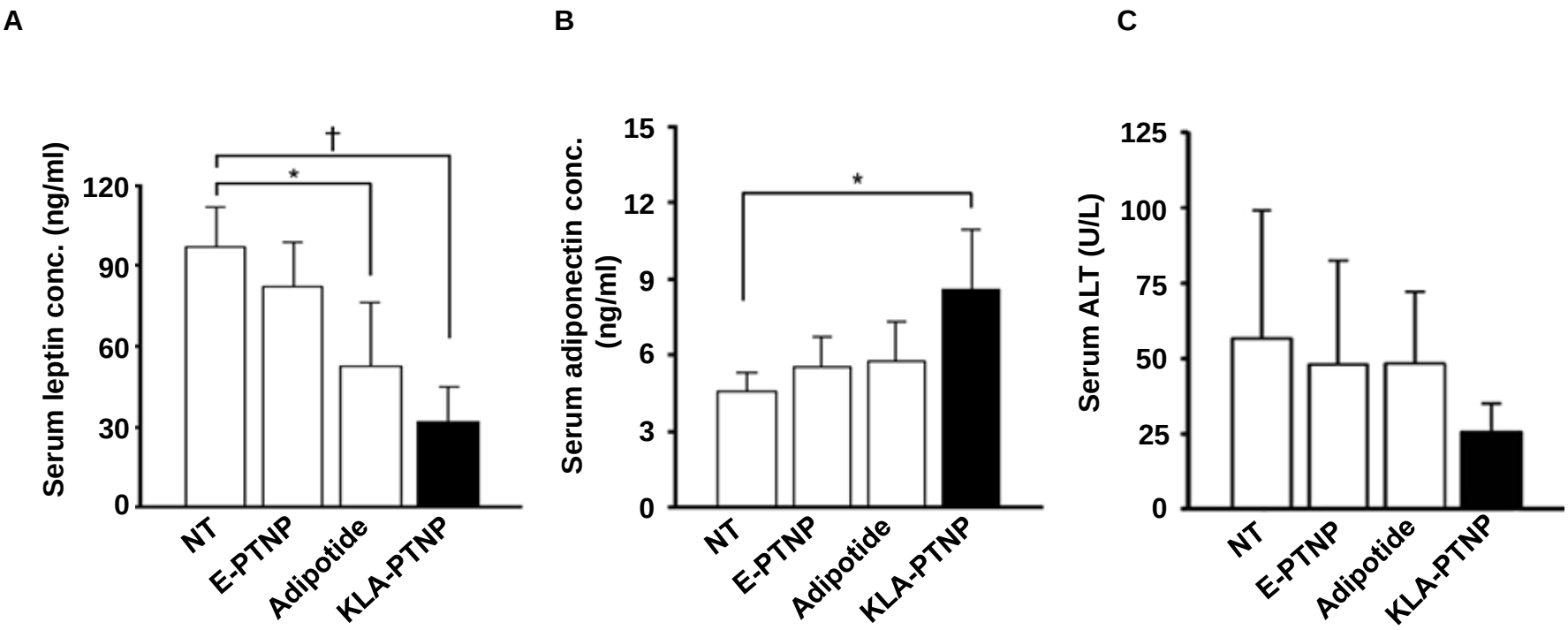
A
B
C
Serum leptin conc. (ng/ml)
120
90
60
30
0
*
†
NT
E-PTNP
Adipotide
KLA-PTNP
Serum adiponectin conc. (ng/ml)
15
12
9
6
3
0
*
NT
E-PTNP
Adipotide
KLA-PTNP
Serum ALT (U/L)
125
100
75
50
25
0
NT
E-PTNP
Adipotide
KLA-PTNP

Fig. 4

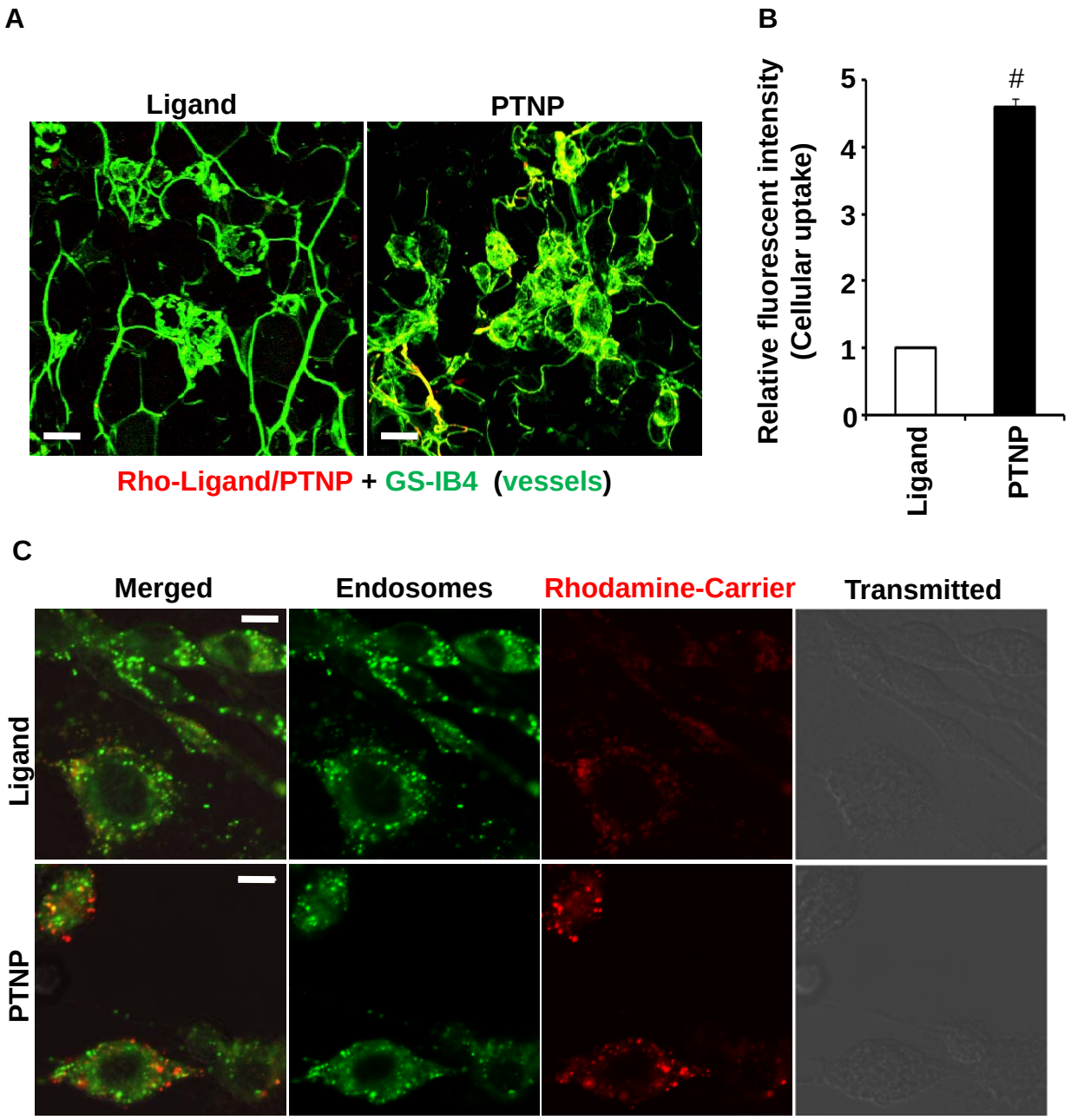
A
Ligand
PTNP
Rho-Ligand/PTNP + GS-IB4 (vessels)
B
Relative fluorescent intensity
(Cellular uptake)
5
4
3
2
1
0
#
Ligand
PTNP
C
Merged
Endosomes
Rhodamine-Carrier
Transmitted
Ligand
PTNP
Rho-Ligand/PTNP + Lysotracker (Endosomes)

Fig. 5

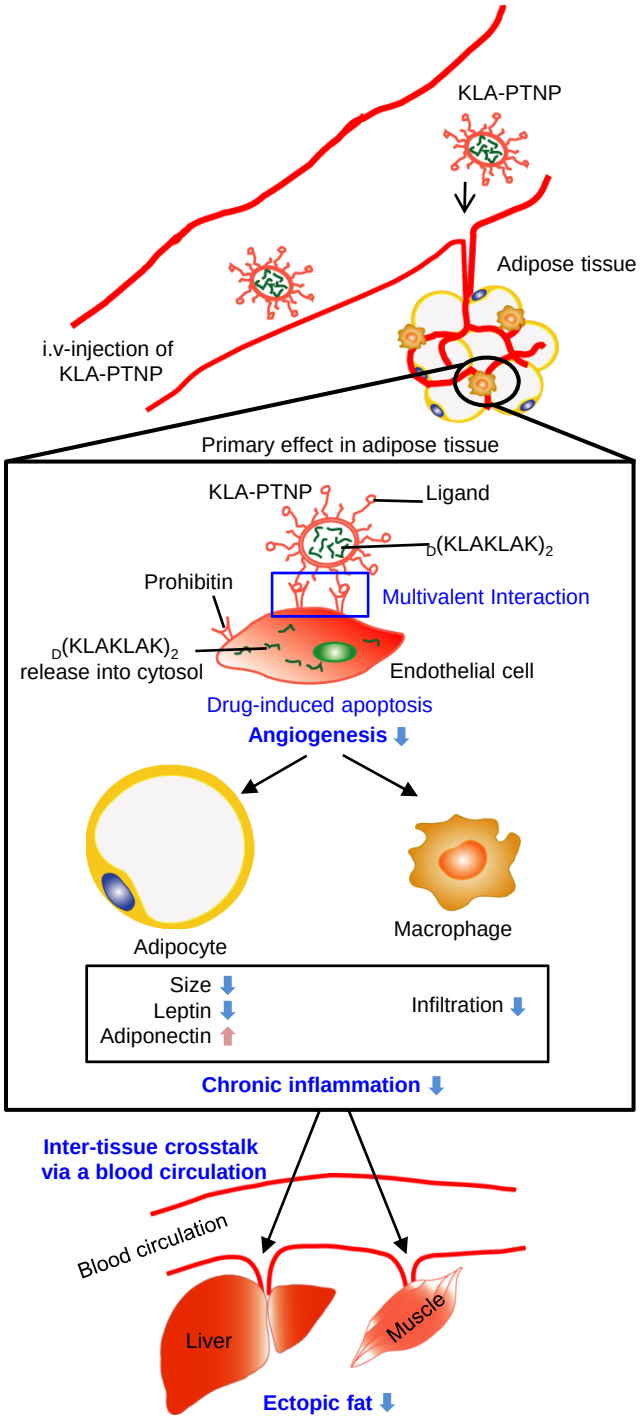
KLA-PTNP
Adipose tissue
i.v-injection of
KLA-PTNP
Primary effect in adipose tissue
KLA-PTNP
Ligand
D(KLAKLAK)2
Prohibitin
Multivalent Interaction
D(KLAKLAK)2
release into cytosol
Endothelial cell
Drug-induced apoptosis
Angiogenesis
Adipocyte
Macrophage
Size
Leptin
Adiponectin
Infiltration
Chronic inflammation
Inter-tissue crosstalk
via a blood circulation
Blood circulation
Liver
Muscle
Ectopic fat

Fig.6