**Tracking adiponectin biodistribution via fluorescence molecular tomography indicates increased vascular permeability after streptozotocin-induced diabetes**

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Keywords: Adiponectin, heart, diabetes, fluorescence molecular tomography, endothelial, vascular permeability

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**Abstract**

Adiponectin, a highly abundant polypeptide hormone in plasma, plays an important role in the regulation of energy metabolism in a wide variety of tissues as well as showing important beneficial effects in diabetes, inflammation and cardiovascular disease. To act on target tissues, adiponectin must move from the circulation to the interstitial space, suggesting that vascular permeability plays an important role in regulating adiponectin action. To test this hypothesis, fluorescently labelled adiponectin was used to monitor its biodistribution in mice with streptozotocin-induced diabetes (STZD). Adiponectin was indeed found to have increased sequestration in the highly fenestrated liver and other tissues within 90 min in STZD mice. In addition, increased myocardial adiponectin was detected and confirmed using Computed Tomography (CT) co-registration. This provided support of adiponectin delivery to affected cardiac tissue as a cardioprotective mechanism. A higher adiponectin content in the STZD heart tissues was further examined by ex vivo fluorescence molecular tomography (FMT) imaging, immunohistochemistry and Western blot analysis. In vitro mechanistic studies using an endothelial monolayer on inserts and 3D microvascular networks on microfluidic chips further confirmed that adiponectin flux was increased by high glucose. However, in the in vitro model and mouse heart tissue, high glucose levels did not change adiponectin receptor levels. An examination of the tight junction (TJ) complex revealed a decrease in the TJ protein claudin (CLDN)-7 in high glucose-treated endothelial cells and the functional significance of this change was underscored by increased endothelium permeability upon siRNA-mediated knockdown of *CLDN-7*. Our data support the idea that glucose-induced effects on permeability of the vascular endothelium contribute to the actions of adiponectin by regulating its transendothelial movement from blood to the interstitial space. These observations are physiologically significant and critical when considering ways to harness the therapeutic potential of adiponectin for diabetes.

**Introduction**

Extensive research on adiponectin has validated this hormone as a biomarker for cardiometabolic diseases and as a therapeutic target with enormous potential ([43](#_ENREF_43)). In order to harness the numerous beneficial effects of adiponectin, it is essential to fully understand the mechanisms governing adiponectin action ([29](#_ENREF_29), [31](#_ENREF_31), [39](#_ENREF_39)). To date, the vast majority of studies have focused on correlating changes in circulating adiponectin levels and disease markers ([1](#_ENREF_1)). As a result, reduced adiponectin levels are well established to inversely correlate with diabetes and cardiovascular diseases ([43](#_ENREF_43)). Adiponectin acts via binding to membrane receptors AdipoR1, AdipoR2 and T-cadherin ([19](#_ENREF_19), [45](#_ENREF_45)), and reductions in their expression or post-translational modification in disease states has been proposed to lead to adiponectin resistance ([11](#_ENREF_11), [36](#_ENREF_36), [42](#_ENREF_42)). However, another critical factor that likely determines adiponectin action is vascular permeability ([25](#_ENREF_25), [26](#_ENREF_26), [46](#_ENREF_46)). More specifically, to directly elicit a response in a target tissue adiponectin must transit from the circulation to interstitial space across the endothelial barrier ([46](#_ENREF_46)).

The monolayer of endothelial cells lining the circulatory system acts as a barrier that regulates the movement of blood borne factors to the interstitial space. In turn, the barrier properties of the endothelium are regulated by the transcellular pathway (i.e. solute transport across endothelial cells) as well as the paracellular pathway (i.e. solute movement between adjacent endothelial cells) ([46](#_ENREF_46)). In general, larger macromolecules move via facilitated transcellular trafficking while the paracellular route typically restricts solutes in the range of 3 nm radius ([16](#_ENREF_16)). Tight junctions (TJ) provide a selective barrier to solute movement between cells and altered expression of components including ZO-1, occludin, tricellulin and claudins alter TJ structure to make a leakier or tighter barrier for paracellular movement of solutes ([15](#_ENREF_15)). Numerous previous studies, particularly focusing on insulin, have shown that vascular permeability can play an important role in contributing to the development of diabetes and heart failure ([25](#_ENREF_25), [46](#_ENREF_46)). It is important to note that endothelial permeability varies widely throughout the body in a tissue (and intra-tissue)-specific manner ([46](#_ENREF_46)). It has also been shown that endothelial barriers control adiponectin transport in a cell- and tissue-specific manner ([35](#_ENREF_35)) and we have reported that transendothelial movement of adiponectin was reduced by glucocorticoids ([9](#_ENREF_9)).

We believe that the role of endothelial permeability as an important determinant of adiponectin action has been somewhat underappreciated. In this study, we further considered a link between vascular permeability, adiponectin flux, and hyperglycaemia/diabetes by producing recombinant adiponectin, which we then conjugated with a near infra-red probe in order to track its biodistribution in live wild type and diabetic mice using fluorescence molecular tomography (FMT). We also examined the effects of high glucose levels on adiponectin flux across isolated arteries and cultured monolayers of endothelial cells. Finally, since the cardioprotective actions of adiponectin are currently the focus in our lab ([6-8](#_ENREF_6), [30](#_ENREF_30), [32](#_ENREF_32)), we considered the role of the vascular barrier and diabetes on adiponectin access and action in the heart. These studies provide a novel and integrated view on the movement of adiponectin across the endothelial barrier under conditions that relate to diabetes and new insight into the impact that vascular permeability has on the actions of adiponectin on key organs.

**Material & Methods**

*Experimental animal model*

Male Nu/Nu homozygous mice and C57BL6 aged 8-13 weeks were utilized for assessing adiponectin biodistribution. All mice were maintained with access to water and low-fluorescent chow (Teklad global 18% Protein Rodent Diet (Irradiated), Harlan Laboratories, Indianapolis, IN, USA) throughout each experimental period. Age and weight-matched pairs were grouped for each injection (averaging 23~25g). After 12 h starvation, diabetes was induced by single intraperitoneal injection of 150mg kg-1 (body weight) STZ (Sigma-Aldrich, St. Louis, MI, USA). Experiments were performed 4 days after STZ injection and diabetes was diagnosed when mice exhibited a blood glucose level > 14mmol/L at this time, whilst all control mice had only saline injection. Circulating adiponectin was detected in serum before and after infusion of VivoTag-750 conjugated adiponectin using a mouse adiponectin ELISA kit (Immunodiagnostics Ltd, Hong Kong) which detects all adiponectin oligomeric forms.

*Fluorescence Molecular Tomography (FMT) to Detect VivoTag-750- adiponectin*

Recombinant adiponectin prepared in our laboratory was labeled with VivoTag-S 750 (NEV10123, Perkin Elmer, Boston, MA, USA). Importantly, this recombinant protein formed oligomeric forms in a ratio which closely mirror those found in circulation (see supplementary figures 1&2). Labeling efficiency was assessed by running labeled full-length adiponectin on a SDS-PAGE gel then scanned on a LI-COR Odyssey infrared imaging system to visualize the 3 adiponectin isoforms. Labeled proteins were transferred from the gels onto nitrocellulose membranes before probing with adiponectin-specific antibodies to assess fluorescence-to-total protein ratio. Labeled adiponectin was infused into lightly anesthetized mice (1-2% isoflurane) via a surgically placed permanent jugular vein cannula. For cannulation insertion, the mice were first anesthetized with 5% isoflurane then maintained at 2% isoflurane. The surgical site was cleaned with iodine solution and alcohol before a longitudinal incision of about 15 mm was made in the skin at the neck of the animal, just above the right front leg. Connective tissue surrounding the jugular vein was carefully separated before two sutures of 6-0 Ethilon surgeon’s silk (Johnson & Johnson Intl, Brussels, Belgium) were placed ~5-10mm apart on the vein. A fine lateral incision was made between the sutures using micro-scissors, which allowed insertion of a saline-filled polyethylene tube (PE10) into the vein between the sutures. When placement was confirmed, the caudal suture was released and the cannula slowly fed caudally ~ 5mm into the vein towards (but not entering) the heart. To confirm correct placement, 50 µl saline was delivered through the cannula using a 31G insulin needle. The cannula was then securely tied to the jugular vein with silk suture and 85 µg of adiponectin conjugated with VivoTag-S was administered followed by a 50 µl saline flush. The cannula was sealed using a heated haemostat, the skin incision was closed using silk suture, and a serum sample was collected from the tail vein within 2 min of adiponectin infusion. Mice were maintained at 2% isoflurane while being positioned in a glass mouse imaging cassette, then scanned 10, 30, 60, and 90min post-adiponectin infusion with a FMT 2500 LX Quantitative Tomography system (VisEn Medical, Perkin Elmer, Downers Grove, IL, USA) using the 750nm near infra-red channel (750/800nm excitation/emission). Once FMT scanning was completed, mice in the imaging cassette were immediately taken for a computed tomography (CT) scan (Locus eXplore MicroCT, GE Healthcare, London, ON, Canada and XRAD 225Cx, Precision X-Ray, North Banford, CT, USA) under constant anesthesia to ensure identical mouse positioning and accurate alignment between CT and FMT. FMT software (TrueQuant, Perkin Elmer) reconstructs a quantitative 3D dataset in which fluorescence/voxel is expressed in nmol/L. FMT and CT datasets (dicom format) were imported into Inveon Research Workplace (Siemens Healthcare, Germany) or AMIDE (http://amide.sourceforge.net) for FMT-CT co-registration to permit accurate localization of the fluorescent signal.

*FMT analysis of tissues ex vivo*

To avoid potential scatter from adjacent tissue, heart, liver, kidney, pancreas and skeletal muscle, those tissues were excised from mice after cervical dislocation immediately following FMT and CT scanning (~100-120min post-adiponectin infusion). 2D epifluorescence FMT images were taken of these tissues on an opaque resin imaging block in the FMT imaging cassette. Quantitative analysis of the fluorescent signal was performed using the FMT software (TrueQuant, Perkin Elmer, Downers Grove, IL, USA).

*Ex vivo vascular permeability of adiponectin* *assay*

Five weeks old Wistar rats were used. After two weeks of standard diet in the animal facility, rats were fasted 5 hours before induction of diabetes by a single intraperitoneal injection of streptozocin (STZ, Sigma-Aldrich, St. Louis, MI, USA) at a dose of 100 mg.kg-1 bodyweight (~200 µl/rat). Diabetes was diagnosed when hyperglycemia was higher than 10 mmol/L (180 mg/dL). Rats developed diabetes within 7 days of STZ injection. A control group of rats was injected with an equivalent volume of the vehicle solution (citrate buffer 0.5M, pH 4.5). Rats that failed to respond to STZ injection were not used for the study. Control or diabetic rats were anesthetized with isoflurane and a mid-line laparotomy was performed to expose and remove the mesenteric bed. Isolated mesenteric arteries were mounted on a glass cannula immerged in a 2-ml organ bath filled with a physiological salt solution (PSS; mmol/L: 130 NaCl; 4.7 KCl; 1.18 KH2PO4; 1.17 MgSO4; 14.9 NaHCO3; 1.6 CaCl2; 0.023 EDTA; 10 glucose; pH 7.4) aerated with 12% O2; 5% CO2; 83 % N2 at 37oC following a method previously described ([33](#_ENREF_33)). Arterial segments were pressurized at 80 mm Hg in no-flow conditions and equilibrated for 30 min before starting experiments. 10 µg/mL adiponectin (200 µl) was added to the arterial perfusate which circulated at a flow rate of 2 µl/min for 90 min at a constant pressure of 80 mm Hg. At 0, 30, 60, 90 min, 100 µl samples of the extra-vascular bath containing transported adiponectin were collected, and an equivalent volume of physiological solution was added into the bath. Adiponectin levels were quantitatively determined by ELISA kit (Immunodiagnostics Ltd, Hong Kong).

*Cell culture*

Human Dermal Microvascular Endothelial Cells HDMEC (ATCC, Manassas, VA, USA) were grown in vascular cell basal medium (ATCC) supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO2. All cells were used at passages 3–4 from the supplier. 500K HDMECs were seeded onto trans-well inserts (Corning, Tewksbury, MA, USA) sized for 12-well plates having 3.0 μm pore sizes. Hyperglycemia was induced with 25 mM glucose in vascular cell basal medium supplemented with 2% FBS for 6 days. To adjust for osmotic pressure differences, control cells were treated with 5.5 mM glucose and 19.5 mM mannitol.

*Measurement of Transendothelial Electric Resistance (TEER)*

Measurements of TEER were conducted using STX-2 chopstick electrodes attached to an epithelial voltohmmeter (EVOM; World Precision Instruments, Sarasota, FL, USA). All TEER measurements were corrected for background by subtracting TEER recorded across a blank membrane primed with appropriate cell culture medium. Resistance measurements were taken at day 0 and day 6 of treatment with 25 mM glucose and control (5.5 mM glucose + 19.5 mM mannitol).

*Permeability Assay using Endothelial Monolayers*

HDMECs were seeded onto trans-well inserts and treated with 25mM glucose and 5.5 mM glucose with 19.5 mM mannitol for 5 days prior to the start of experiments. At the start of the experiment, 10μg/mL of adiponectin with the 25mM glucose or 5.5 mM glucose with 19.5 mM mannitol including 2% FBS vascular cell basal medium (ATCC PCS-100-030, Cedarlane, Burlington, ON, CA) was applied to the apical chamber only. After 24 h, apical and basolateral media were assessed for adiponectin concentration by gel electrophoresis after concentrating with 10,000Da MWCO (molecular weight cutoff) filter (EMD Millipore, Billerica, MA, USA) or by fluorescence intensity reader, respectively, and concentrations calculated by comparison to standard curves prepared in culture medium.

*Quantitative RT-PCR*

Using aliquots of total extracted RNA, cDNA was synthesized. And pRT-PCR reactions were performed using the SYBR Green PCR Master Mix (Lifetechnologies). Gene-specific primer sets were designed. human CLDN7, F: 5’ GGAGACGACAAAGTGAAGAAGG 3’, R: 5’ GGACAGGAACAGGAGAGCAG 3’, human GAPDH, F: 5’ AACATCATCCCTGCCTCTACTG 3’, R: 5’ CCTGCTTCACCACCTTCTTG 3’, murine AdipoR1, F: 5’ ACGTTGGAGAGTCATCCCGTAT 3’, R: 5’ CTCTGTGTGGATGCGGAAGAT 3’, murine AdipoR2, F: 5’ TCCCAGGAAGATGAAGGGTTTAT 3’, R: 5’ TTCCATTCGTTCCATAGCATGA 3’, murine Adiponectin, F: 5’ TGTTCCTCTTAATCCTGCCCA 3’, R: 5’ CCAACCTGCACAAGTTCCCTT 3’, murine CLDN7, F: 5’ GGACCTGCCATCTTTATCGGC 3’, R: 5’ AGCTTTGCTTTCACTGCCTGG 3’, murine ZO-1, F: 5’ GTCCCTGTGAGTCCTTCAGCTG 3’, R: 5’ ACTCAACACACCACCATTGCTG 3’, murine 18s RNA, F: 5’ AGTGAAACTGCGAATGGCTCA 3’, R: 5’ CGAGCGACCAAAGGAACCA 3’.

*Claudin-7 Knockdown by shRNA in HDMEC*

Claudin-7 gene expression was knocked-down in HDMECs using a pGPU6/Neo-claudin-7 plasmid as shown previously ([9](#_ENREF_9)) using the following shRNA target sequence: 5′-GGCCATCAG ATTGTCACAGAC-3′ (GenePharma Co., Ltd., Shanghai, China). Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA) was used for all transfections following manufacturer instructions. A non-targeted control (NTC) shRNA plasmid was designed with a non-specific scrambled sequence. The transfected cells were stabilized for 24h with 10% FBS medium and subjected to antibiotic selection (500 μg/mL G418 (Sigma-aldrich, Saint Louis, MO, USA)) for 2 days. During antibiotic selection, transfected cells were seeded on trans-well inserts or 6 well-plates for further experiments.

*Western Blotting*

Proteins from mouse tissues were homogenized by TissueLyser II (QIAGEN, Hilden, Germany) in RIPA buffer with 80mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 20% glycerol, 3.3% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue, 30mM Hepes (pH 7.4), 2.5mM EGTA, 3mM EDTA, 70mM KCl, 20mM β-glycerolphosphate, 20mM NaF, 1mM Na3VO4, 200μM PMSF, 10μM E64, 1μM leupeptin, 1μM pepstatin A, 0.1% NP40, and 0.1μM okadaic acid. To detect the 3 forms of adiponectin, samples were prepared without β-mercaptoethanol or heating. For detection of other protein targets, samples were lysed in complete RIPA buffer and denatured at 95°C for 10min. Transported adiponectin was collected from basolateral HDMEC medium and concentrated using Amicon Ultra-4 Centrifugal Filter Units with Ultracel-10K (EMD Millipore, Billerica, MA, USA). To detect the 3 adiponectin oligomers, concentrated adiponectin samples were prepared without denaturation or reduction. Prepared samples were run on SDS-PAGE gels then transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules CA, USA), before incubation with primary antibody, and detection by chemiluminescence. Polyclonal primary antibody of rabbit anti-Adiponectin (dilution 1:1000) is produced in-house ([28](#_ENREF_28)). Rabbit anti-AdipoR1/2 antibodies were from Phoenix Biotech (Toronto, ON, CA) ([32](#_ENREF_32)). Mouse anti-FLAG M2 (dilution 1:1000, F1804, Sigma, Oakville, ON, CA), rabbit anti-Claudin-7 (dilution 1:500, Cat#34-9100, Thermo Fisher, Rockford, Illinois, USA), rabbit anti-α/β-Tubulin (dilution 1:1000, Cat#2148, Cell Signaling, Danvers, MA, USA), rabbit anti-GAPDH(14C10) (dilution 1:1000, Cat#2118, Cell Signaling, Danvers, MA, USA) were purchased. And the appropriate HRP-conjugated secondary antibody (anti-rabbit IgG-HRP (dilution 1:5000, Cat#7074), anti-mouse IgG-HRP (dilution 1:5000, Cat#7076)) was used from Cell Signaling (Danvers, MA, USA). Bands were quantified by densitometry using Fiji software and normalized to relevant loading controls as indicated.

*Immunofluorescence*

Mouse hearts were isolated following isoflurane anesthesia and the ventricles excised then cross-sectioned at the mid-line with a surgical blade before being embedded into a mold with Optimal Cutting Temperature compound (O.C.T) (Sakura Finetek USA Inc., Torrance, CA, USA) and frozen on dry ice. 5 μm thick cryosections were made using a cryostat and mounted onto positively charged glass slides (Superfrost, ThermoFisher, CA). Mounted slides were fixed in 4% PFA solution for 10min to stain adiponectin or fixed in 100% ice-cold acetone, air-dried at room temperature, and rehydrated in distilled water followed by washing with phosphate-buffered saline (PBS) buffer. And permeabilization was followed by 0.5% TritonX-100. Before permeabilization, cell border staining was performed by incubating with 5 µg/mL Alexa488 conjugated wheat germ agglutinin (Thermo Fisher, Eugene, OR, USA) for 10min in adiponectin staining. Sections were then incubated with Adiponectin primary antibody (1:100) and Texas-Red secondary antibody (1:250) and DAPI for nuclear visualization. For vasculature staining, primary antibodies directed against Claudin-7 (dilution 1:50, Cat#34-9100, Thermo Fisher, Rockford, Illinois, USA), VE-Cadherin (dilution 1:100, Cat#PA5-17401, Thermo Fisher, Rockford, Illinois, USA), PECAM-1 (dilution 1:50, Cat#SC-376764, Santa Cruz Biotech, Mississauga, CA), ZO-1 (dilution 1:100, Cat#61-7300, ThermoFisher Scientific, Rockford, Illinois, USA), α-SMA (1:100, α-SM1, a kind gift from Dr. Giulio Gabbiani, University of Geneva, Switzerland), and desmin (1:30, Cat#M076029, Dako, Burlington, ON, CA) were used. Isotype-specific secondary antibodies anti-rabbit IgG-TRITC (1:100, Sigma, St. Louis, Missouri, USA), Alexa 568-conjugated IgG (1:100, Abcam, Carlsbad, CA, USA), Alexa647-conjugated IgG2a (1:100, Molecular Probes, LifeTechnologies Inc., Rockford, Illinois, USA), and anti-mouse IgG1-FITC (1:200, SouthernBiotech, Birmingham, Alabama, USA), and DAPI (Vectashiled mounting medium with DAPI, Cat# H-1200, Burlingame, CA, USA) were used. Images were acquired using a LSM700 (Zeiss) or LSM780 (Zeiss) confocal microscope and an upright Zeiss Axio Observer M35 epifluorescence microscope equipped with structured illumination (Apotome) and Axiocam HR camera (Carl Zeiss, Jena, Germany). All images were processed using Fiji or Adobe Photoshop CS5 (Adobe System, San Jose, CA). Contrast and brightness were enhanced consistently for all representative images used in the publication.

*Fabrication of Microfluidic Devices and Cell culture on a Chip*

To recapitulate 3D microvascular networks *in vitro*, we utilized a microfluidic chip that can include cells-trapped hydrogel and cell growth medium with compartmentalization (a central gel channel and two lateral media channels). Previous studies have described the fabrication process of the chips in detail ([37](#_ENREF_37)). Briefly, silicon-based organic PDMS microfluidic device fabrication includes steps of soft lithography with SU-8 silicon wafer mold (AMED Inc.) and PDMS (1:10, Dow Corning), generating reservoirs with 1, 4 mm diameter biopsy punches (KAI medical), and device assembly by plasma bonding (Femto Science Inc.) to a coverslip (glass, Duran). The devices were sterilized by 15min sonication (Uil Ultrasonic Co., Ltd) in 70% EtOH before the bonding process. An 80°C drying oven was used for curing PDMS mixture, dehydration of devices before plasma treatment and recovering hydrophobicity after bonding (24hr in advanced cell seeding). In this study, 120μm depth of microchannel devices was used.

Human Umbilical Vein Endothelial Cells (HUVECs, Lonza) were grown in endothelial cell growth medium (EGM-2, Lonza) supplemented with 5% fetal bovine serum (FBS) at 37°C, 5% CO2. All cells were used at passages 6-8 from the supplier. After harvesting of trypsinized cells by centrifugation (125 xg, 7 min), cells were resuspended in concentration of 1.4 x 107 cells/mL with 4U/mL thrombin solution. We injected 1:1 mixture of cells (1.4 x 107 cells/mL) and fibrinogen solution (5 mg/mL) into a gel inlet to fill it up. After incubating the chips at 37 ° C in a humid chamber for 15 minutes to wait gelation, we injected cell culture medium into the media channels. The cells in the microfluidic chips were incubated for 4 days with daily replacement of medium. Self-assembly of HUVECs into a perfusable vascular network (vasculogenesis) was induced by adding 50 ng/mL VEGF to EGM from day 0 (D0) to day 4 (D4).

*Vascular Permeability Assay in Microfluidic Chips*

After 4 day culture, medium was washed out with a quick PBS rinse before a fluorescent molecule solution was infused. Due to the hydrostatic pressure drop between the two media channels, the solution in the media channel flows along with the perfusable microvascular networks to the opposite media channel. We monitored the diffusion of fluorescent molecules into the fibrin gel (ECM) through the vessel wall and the images were captured at 6-10 second intervals for 3 min via a fluorescence microscope (Axio Observer Z1, Zeiss). The images were quantified using ImageJ.

*Perfusibility test in 3D microvascular network*

At day 4, endothelial growth medium was quickly washed with PBS by filling up two reservoirs in only one side channel to rinse out any particles from the 3D vessel lumen efficiently using hydrostatic pressure differences. When a desired field of view was found, bright field images were captured. Then, 5.0-5.9 μm of FITC surface labeled beads (0.1% w/v, F1CP-50-2, Spherotech) was infused into one media channel and time-lapse images were taken at 1.4 second intervals for 2 min using the fluorescence microscope (Axio, Observer Z1, Zeiss). The fluorescent images were overlayed with brightfield orientation image, and processed to video with ImageJ.

*Statistical analysis*

Data are expressed as mean ± SEM and the significant differences was determined where p<0.05 resulted from performing statistical analysis with Student’s t-test or multiple t-test and one-way ANOVA.

**Results**

*Diabetes increases biodistribution of NIR-labeled full-length adiponectin into target tissues*

To accurately investigate adiponectin biodistribution, we developed a model using fluorescence molecular tomography (FMT) in which 3-dimensional localization of fluorescently tagged adiponectin could be tracked in short time intervals *in vivo*. Full-length adiponectin comprising of high, medium and low molecular weight oligomers was tagged with a near-infrared (NIR) peptide (VivoTag-S 750) detectable at 750nm (fAd-VT750). This wavelength, and feeding mice with AIN-76A low-chlorophyll chow diet (Harlan, Indianapolis IN), allowed us to avoid confounding effects of autofluorescence ([2](#_ENREF_2)). Western blot and fluorescent imaging confirmed that all 3 oligomers of adiponectin were fluorophore conjugated (Supp 1A), and we demonstrated a linear relationship between adiponectin concentration and fluorescence measurement (Supp 1B, C).

To test the hypothesis that hyperglycemia could alter adiponectin biodistribution, we infused fAd-VT750 (85µg, via jugular vein) into STZ injected (STZD) or control nu/nu mice, then monitored whole body fluorescence in mice by FMT imaging. We found no difference in circulating adiponectin levels between control and STZD mice and also observed the same level of fAd-VT750 in serum of either STZD or wt mice 2 min after injection via jugular vein (Supp 2A, B). Consecutive FMT images at 0, 10, 30, 60 and 90 min after fAd-VT750 injection showed increasing tissue accumulation of fluorescent adiponectin throughout the body which appeared to be faster or higher in STZD mice (Fig 1A-C), particularly showing enhanced liver and/or heart accumulation relative to controls. FMT imaging confers the added advantage of providing quantitative analysis of whole body fluorescence and clearly measured an increased overall fluorescence intensity in STZD mice. As a control, infused unconjugated VT750 displayed a completely different biodistribution pattern, clearing quickly through the kidneys and localized primarily to the bladder within 60 min, with very little signal coming from other peripheral tissues (Supp 3).

To conduct further analysis of the tissue localization of fluorescent signal, mice also underwent x-ray computed tomography (CT) imaging for fluorescence co-registration (Fig 1D). Coronal and sagittal sections of FMT/CT co-registration data showed accumulation of fAd-VT750 in the heart, liver, kidney, and bladder (Fig 1E). To eliminate potential tissue scattering effects, major target tissues of adiponectin action were removed 90 min following fAd-VT750 infusion and imaged ex-vivo by FMT. Heart, liver, kidney, pancreas, and skeletal muscle (Sk M) of STZD mice exhibited a higher fluorescent signal when compared to wt (Fig 1F), although only heart and liver reached significance upon quantitation (Fig 1G). In agreement with this, Western blot analysis of reduced tissue homogenates, to allow detection of all adiponectin as monomers, clearly showed increased levels in all of these target tissues from STZD versus wt at 90min post fAd-VT750 infusion (Fig 1H).

*Adiponectin accumulation in the heart is upregulated by STZD.*

Because the main research focus of our laboratory is cardioprotective effects of adiponectin, we then used FMT-CT co-registered images to quantify the fluorescence contained within a 3-dimensional region of interest mapped to the heart (Fig 2A). FMT quantitation shows that within 10 minutes fAd-VT750 level in STZD hearts was significantly greater than in wt (Fig 2B). Accordingly, area under the curve analysis showed greater accumulation of fAd-VT750 in the hearts of STZD mice (Fig 2C). Immunofluorescent analysis of cross-sections from myocardium also indicated increased fAd-VT750 in STZD mice 90 min post-infusion compared to wt (Fig 2D,E). Interestingly, higher magnification revealed an apparent co-localization of fAd-VT750 with wheat germ agglutinin (WGA), suggesting a near-membrane location.

Since molecular weight could be an important determinant in the movement of the adiponectin isoforms from circulation into the heart, we assessed the amount of fAd-VT750 in perfused heart homogenates from control and STZD mice under non-denaturing conditions. There was an increased presence of all three oligomeric adiponectin isoforms in STZD heart homogenates when compared to wt (Fig 2G). To verify that increased fAd-VT750 localization was not influenced by presence of the VivoTag-750 fluorophore, Flag-tagged and non-fluorescent recombinant adiponectin was injected into wild-type C57BL/6 mice treated with or without STZ. Similar to fAd-VT750, there was increased total heart localization of Flag-adiponectin in STZ treated mice compared to controls (Fig 2F). Quantitative PCR analysis showed no difference in adiponectin mRNA levels between control and STZD cardiac homogenates (Fig 2H).

*High glucose levels regulate claudin-7 expression to increase paracellular movement of adiponectin.*

We next tested vascular permeability of adiponectin using mesenteric arteries isolated from STZD or control mice. Similar to our *in vivo* findings, adiponectin flux across the arterial wall of STZD mesenteric arteries was greater compared to wt (Fig 3A). We then exposed a monolayer of human dermal endothelial cells (HDMECs) cultured on trans-well inserts to high glucose and found that flux of adiponectin, added to apical chamber, was higher than in normal glucose conditions. Fig 3B shows a Western blot, with quantitation below, indicating a significant increase in flux of HMW-, MMW-, and LMW-adiponectin from apical to basolateral side in cells grown under hyperglycemic conditions (Fig 3B). Immunoblotting of the adiponectin receptors AdipoR1 and AdipoR2 showed that their expression in HDMEC monolayers was not affected by high glucose (Supp 4C). Accordingly, we found that endothelial barrier tightness measured by trans-endothelial electrical resistance (TEER) was decreased in high glucose treated cells (Fig 3C). This corresponded to a decrease in claudin-7 (cldn-7) mRNA and protein expression following high glucose treatment (Fig 3D, E). Immunofluorescence imaging indicated a decrease in cell membrane expression of cldn-7 in high glucose treated cells compared to control (Fig 3F), whereas there was no difference in expression of another tight junction related protein, ZO-1, between groups (Fig 3G). This observation was mirrored when heart homogenates were analyzed. Protein and mRNA levels of adiponectin receptors were unchanged by STZD (Supp 4A,B). In cardiac tissue sections analyzed by qPCR for mRNA and immunohistochemistry, ZO-1 level was not significantly lower (Fig 3J, K) while cldn-7 expression was significantly decreased (Fig 3H, I) in STZD hearts compared to wt.

*Reduced claudin-7 expression decreased TEER and enhanced adiponectin movement across endothelial monolayer.*

To test the functional importance of cldn-7 in adiponectin flux across an HDMEC monolayer, knockdown of cldn-7 was induced by transfection of shRNA (shCLDN7), which resulted in approximately 50% decrease in cldn-7 protein compared to cells that received a scrambled shRNA sequence not targeting cldn-7 (shNTC) (Fig 4A). TEER was significantly decreased in monolayers comprised of shCLDN7 cells compared to shNTC (Fig 4B). Furthermore, there was increased flux of LMW-adiponectin from apical to basolateral side of monolayers comprised of shCLDN7 cells compared to shNTC controls (Fig 4 C).

*Functional 3D perfusable microvascular networks allowed to confirm hyperglycemia effect on permeability of dextran and adiponectin*

We finally tested the hyperglycemia effect in microfluidic microvascular network of HUVECs (human umbilical vein endothelial cells). This in vitro platform of 3D vasculature-on-a-chip was generated in the ECM mimetic environment with fibrin hydrogel.PDMS (polydimethylsiloxane) microfluidic device is useful to induce perfusable microvasculature as well as to monitor the event inside of the highly branched vascular structure. The PDMS mold has a central gel channel containing HUVECs and fibrin hydrogel bordered by triangular posts and two media fluid channels (Fig 5A, B). 1.4 x 107 cells/mL of HUVECs were seeded with the fibrin hydrogel at D0 and incubated for 4 days to form endothelial monolayers of in vivo-like vasculature naturally (Fig 5C). Additional VEGF was used to increase the dynamic alignment of HUVECs from D0 to D4 (Fig 5D). At day 4, the formation of open-ended EC monolayer 3D vessels between two PDMS posts were completed. GFP transfected HUVECs showed highly branched features of microvascular networks in a device (Fig 5E) and vascular endothelial cadherin (VE-Cadherin) positive stained structures indicated that vessel-like EC monolayer barrier allows the perfusion of fluids (Fig 5F, Sup Video 3). To confirm the functionality of this vasculature, we performed perfusibility test with 5 μm beads conjugated with FITC and timelapes images were taken under fluorescence exposure at every 1.4 sec (Fig 5G, Sup Video 4). Next, we wanted to test the effect of hyperglycemia on permeability of 3D microvascular networks using full length adiponectin (fAd) and changes of paracellular movement across of this barrier with 3, 70kDa dextran. Immunofluorescent detection of VE-C showed that the integrity of interconnected vascular structures appeared not to loosen in hyperglycemia compared to control (Sup Fig 5). To assess the permeability, 10 μg/mL fAd conjugated with rhodamine was injected into one reservoir of a device and fluorescent images were captured overtime. The apparent permeability (Papp, cm/sec) was calculated by the fluorescent intensity measurement from a linear ROI spanning the microvessel-ECM interface. Because the perfused fluorescent molecules were transmoved to ECM from the vessels by concentration gradient, the intensity increase was observed in ECM area overtime (Fig 5H). Permeability of rhodamine labeled fAd (Rho-fAd) increased in hyperglycemia (1.285 x 10-5 ± 0.09363 x 10-5 cm/sec) compared with 19.5mM mannitol with 5.5mM glucose treated control (2.290 x 10-5 ± 0.1651 x 10-5 cm/sec) (Fig 5K). 70kDa Dextran which is smaller than LMW adiponectin (around 90kDa) also showed significantly higher permeability in high glucose treated condition (Papp of HG was 6.812 x 10-5 ± 1.152 x 10-5 cm/sec and control Papp was 2.180 x 10-5 ± 0.2277 x 10-5 cm/sec) (Fig 5J). However, for the small size of dextran (3kDa-FITC), hyperglycemia did not significantly change the permeability (Fig 5I). These suggested that paracellular movement regulates transendothelial accessibility depending on the size of molecules. And it confirmed that hyperglycemia *in vitro* with 25mM glucose altered the movement of full-length adiponectin in microfluidic vessels-on-a-chip.

**Discussion**

Adiponectin has been shown to mediate widespread physiological effects with resulting anti-diabetic, anti-inflammatory and cardioprotective effects ([12](#_ENREF_12)). Hence, there has been great interest within academic institutes and pharmaceutical companies to develop therapeutics targeting adiponectin action. To accomplish this, it is critical to fully understand the mechanisms regulating adiponectins cellular effects in various tissues. Whilst we have learned much about adiponectin receptor mediated signaling mechanisms, few studies have focused on examining mechanisms regulating the transit of adiponectin from the circulation to the interstitial space, and thus target cells such as cardiomyocytes, hepatocytes, -cells and other cells. We previously proposed that vascular permeability was likely to be an important, and underappreciated, determinant of adiponectin’s physiological actions ([46](#_ENREF_46)). This is particularly relevant since adiponectin is a complex molecule comprised of multiple oligomeric forms, each with a different molecular weight and radial size estimated to be 3.96 to 10.1 nm ([35](#_ENREF_35)). This is exactly the range within which tight junctions of around 4nm in dimension can be expected to strongly contribute to dictating adiponectin flux. Indeed, we recently demonstrated that glucocorticoid-mediated decreases in transcellular endothelial permeability restricted adiponectin transit across endothelial monolayers ([9](#_ENREF_9)). The observation that adiponectin in cerebrospinal fluid is almost completely in the small trimeric form, and the total amount is only 0.1% of that in peripheral circulation ([27](#_ENREF_27)), suggests that the tight blood brain barrier restricts movement of adiponectin oligomers. Conversely, many studies focusing on metabolic effects of adiponectin have focused on liver, which has a highly fenestrated leaky vasculature, and found that the high molecular weight adiponectin is highly bioactive and correlates well with clinical readouts of liver function ([5](#_ENREF_5), [38](#_ENREF_38)).

In this study, we used a real time non-invasive approach to monitor and quantify the biodistribution of exogenously administered fluorescent adiponectin. The use of FMT as a non-invasive approach to examine biodistribution of a circulating hormone such as adiponectin has strong advantages ([40](#_ENREF_40)). The ability to monitor individual mice non-invasively for time-course studies vastly reduces the number of animals required and avoids issues such as inter-animal metabolic variation and variability in fluorophore injection. In addition, time and expense of isolating numerous tissues followed by analysis by western blot can be a significant limiting factor to the identification of novel target tissues. Having a clear, temporal, whole-body indicator of kinetics can provide a wealth of mechanistic insight, potentially leading to new therapeutics. In this study, we complemented FMT imaging with CT, which offered us high-resolution anatomical detail to improve the spatial and temporal localization of adiponectin. Analysis of co-registered FMT-CT data improves upon methodologies which offer only 2D fluorescence acquisition. Through 3-dimensional analysis, we were able to precisely quantify fAd-VT750 in the heart through creation of CT-guided 3D regions of interest (ROIs). This is vital for accuracy as many factors can impair rough localization of a fluorophore including: mouse orientation, variability in organ size, potential “bleed over” from highly targeted tissues (e.g. liver), and spatial overlap of several tissues such as the lungs, heart and thymus, from certain mouse orientations. Analysis of our *in vivo* findings were substantially corroborated by *ex vivo* scanning of target tissues and classic molecular biology techniques including western blot analysis and immunofluorescence imaging. For fluorescence *in vivo* imaging of whole body biodistribution, it is important to avoid autofluorescence which requires near-infrared (NIR) labeling of a target ([2](#_ENREF_2)). Probes and fluorophores are available in this range, from 680 to 750nm and 800nm, offering the potential for some creative multiplexing studies such as co-tracking of insulin and adiponectin in circulation under various disease models. Imaging at 750-800nm wavelengths completely avoids autofluorescence, and imaging at 680nm only requires the use of low-chlorophyll chow to minimize confounding gastrointestinal background fluorescence.

Using this approach, we studied mice with or without streptozotocin-induced diabetes. Interestingly, an accumulation of adiponectin occurred in the peripheral tissues, particularly liver, and to a greater extent in STZD mice. This was confirmed by quantitative analysis of in vivo FMT images, ex vivo imaging and by analyzing tissue homogenates by Western blot. It was also interesting to note that we found no difference in endogenous adiponectin between control and STZD animals. As we are interested in the cardioprotective effects of adiponectin([11](#_ENREF_11), [14](#_ENREF_14)) when we focused on myocardial adiponectin we again found greater levels in STZD mice. The increased total amount of fAd-VT750 appeared to be accounted for by MMW, plus HMW and to a lesser extent LMW oligomeric forms. It is worth noting that the relative tightness of an endothelial barrier, whether *in vivo*, *ex vivo*, or *in vitro*, and the amount of change induced by a given treatment (e.g. high glucose), can lead to seemingly contradictory results such as the data presented here (Fig 2E vs Fig 4C). This may be explained through an understanding of protein flux dynamics at the cell junction level: slight opening of a tight endothelial barrier may lead to increased flux of only low-molecular weight proteins, while with a leakier barrier, the flux of low-molecular weight proteins may already be maximum. In this case, further opening of a leaky barrier would manifest as increased flux of only higher-molecular weight proteins. STZD is a common diabetic animal model yet data must be interpreted with caution since numerous endogenous changes beyond hyperglycemia may impact vascular permeability ([3](#_ENREF_3), [4](#_ENREF_4), [34](#_ENREF_34), [42](#_ENREF_42)). It would be of great interest to examine the same phenomenon studied here in models such as high fat diet feeding or in genetically obese models such as *ob/ob* mice ([22](#_ENREF_22)). Nevertheless, to extend our observations further we examined flux of adiponectin upon addition to isolated mesenteric vessels from wt or STZD animals. We observed again that vascular permeability of adiponectin was higher in STZD vessels. Furthermore, using an endothelial cell monolayer system *in vitro* we found that flux of adiponectin was significantly greater in monolayers cultured in high glucose media versus normal glucose. Collectively, the data suggests that in the STZD model hyperglecemia is likely to be one of the principal determinants of altered vascular permeability of adiponectin.

In this study we also focused on alterations in tight junction proteins as a potential mechanism underlying the effects observed here. We focused on claudin-7 based on rationale from previous studies which showed that shRNA-mediated reduction in endothelial cell claudin-7 levels increased flux of adiponectin across endothelial monolayers ([9](#_ENREF_9)). Indeed, our data indicated that a decrease in claudin-7 may be one important alteration leading to incresed vascular permeability in STZD mice. Furthermore, we found ZO-1 was not significantly changed in STZD hearts although previously, hyperglycemia was shown to increase permeability of the blood brain barrier ([17](#_ENREF_17)), and retinal pigment epithelial cells ([17](#_ENREF_17), [41](#_ENREF_41)) through downregulation of occludin and ZO-1. Since AdipoR-mediated endocytosis has been shown to have important cellular signaling consequences we also believed that this may contribute to a transcellular route of transport ([10](#_ENREF_10), [46](#_ENREF_46)). However, our data in supplementary figure 4 showing no significant change in the level of cardiac AdipoR1 or AdipoR2 after hyperglycemia in mice or high glucose level in HDMEC, suggests this is likely not a major player.

We used advanced *in vitro* platform of 3D microvascular networks on a chip to confirm the observation from 2D transwell endothelial monolayer responses in high glucose environment. Generating endothelial monolayer of 3D vessels with multiple branches using programmed cellular dynamics in hydrogel allows to mimic better *in vivo* vascular morphology ([23](#_ENREF_23)). Another strength of the 3D microvasculature in microfluidic devices is having perfusibility, which allows to test the functionality of the vessels on a chip as well as to better mimic physiological environment with application of fluidic shear stress ([21](#_ENREF_21), [24](#_ENREF_24), [44](#_ENREF_44)). Using paracellular tracers, many literatures have shown that the basal permeability of comparably large molecules (40, 70kDa dextran) was lower than the permeability of smaller one (4, 10kDa dextran) ([13](#_ENREF_13), [18](#_ENREF_18), [20](#_ENREF_20)). In this study using the platform, we confirmed size-selective transport behavior and showed for the first time that movement of rhodamine labeled full-length adiponectin was significantly impacted by hyperglycemia-mediated alteration of endothelial permeability.

In summary, we have shown that biodistribution of adiponectin can be altered in a diabetic environment. Data from a combination of temporal non-invasive imaging in mice, isolated vessels *ex vivo* and *in vitro* endothelial cell monolayers suggest that hyperglycemia increased vascular flux of adiponectin. We believe this may be an important determinant of adiponectin’s physiological actions and could provide rationale for further investigation and be exploited from a therapeutic perspective.

**Acknowledgements**

This work was supported by a grant to GS from Natural Sciences and Engineering Research Council (NSERC) and Canadian Institutes of Health Research (CIHR). GS also acknowledges support from the Heart & Stroke Foundation via a Career Investigator Award. The research of BH was supported by the Canadian Institutes of Health Research (CIHR), grant #375597, the Canadian Foundation for Innovation (CFI, and the Ontario Research Foundation (ORF), grant#36050. JSJ acknowledges support from Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2017R1D1A1B03030428).

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**Figure Legends**

**Figure 1. Fluorescence molecular tomography shows *in vivo* biodistribution of full-length adiponectin is increased in diabetic conditions.** (A) 85 µg of near-infrared labeled full-length adiponectin (fAd-VT750) was injected via cannulated jugular vein into control or diabetic (STZD) nu/nu mice. Representative images from a control and STZD mouse before injection and at 10, 30, 60, and 90 minutes post-injection are shown. (B, C) Total signal intensity from whole body 3D field of view were calculated as nM (n≥8, \*p<0.05 from multiple t-test). Time course data was converted to area under curve (AUC) (\*p<0.05 from student t-test). (D) Co-registration of FMT (colour) and computed tomography (CT) data (white/grey). (E) Coronal and sagittal view of FMT-CT co-registration with organs of interest identified. (F) Ex-vivo 2D FMT analysis of fAd-VT750 from isolated tissues (heart, liver, kidney, pancreas and skeletal muscle (tibialis anterior)). (G) Quantification of these 2D images using TrueQuant software. \* indicates p < 0.05, compared to its control (n≥4). Statistical analysis performed using student’s t-test. (H) Cardiac homogenates from control and STZD mice were analyzed by Western Blot under non-denaturing conditions to compare total amount of reduced (i.e. monomeric, 30 kDa) adiponectin.

**Figure 2. Adiponectin uptake into the heart is increased by diabetes.**

(A) Representative heart regions of interest (ROIs) generated by alignment of CT and FMT 3D images using IRW software. (B) Quantitation of fAd-VT750 within mapped ROIs. Fluorescence intensity of localized fAd-VT750 (nM) was calculated over time by FMT-CT co-registration analysis (n≥8). \* indicates p<0.05 with respect to control at each time point and the p values were from the multiple t-test. (C) Area under curve analysis of fAd-VT750 within the heart ROI (\*p<0.05) (D, E) Immunofluorescence analysis of transverse heart cryosections stained with adiponectin (red), Wheat Germ Aglutinin (WGA, green), and DAPI (blue). Co-localization of adiponectin and WGA appear orange. (F) Cardiac homogenates from C57BL/6 mice 90 minutes following Flag-tagged adiponectin infusion, run under non-denaturing conditions by SDS-PAGE to detect different adiponectin oligomers: High-Molecular Weight (HMW, >250 kDa), Mid-Molecular Weight (MMW, ~180 kDa), and Low-Molecular Weight (LMW, ~90 kDa). Total adiponectin combines densitometry from all three oligomers, adjusted to GAPDH as loading control (n=5, \*p<0.05). The quantification analyzed by multiple t-test. (G) Detection of fAd-VT750 by SDS-PAGE in heart homogenates from nu/nu mice 90 min post fAd-VT750 infusion. (H) Real-time quantitative PCR analysis of heart homogenates 90mins post-injection adjusted for GAPDH (n=5).

**Figure 3. Hyperglycemia reduces Claudin-7 expression and permits greater flux of adiponectin movement across the endothelium.** (A) Mesenteric arteries extracted from control or STZD rats were perfused with flag-tagged fAd, with samples of extravascular fluid taken at 30, 60, and 90 minutes post adiponectin and analyzed by ELISA. AUC analysis shows greater adiponectin flux from STZD arteries vs control (n≥5, \*p<0.05). (B) HDMEC cells were cultured in a transwell insert before treatment with high glucose (HG) or control. Adiponectin was added to the apical surface then basolateral media was collected 24hr following adiponectin administration. Samples were run under non-denaturing conditions. (n=3, \* indicates p<0.05 with respect to each adiponectin isomer’s control.) (C) TEER (transendothelial electrical resistance) was lower in hyperglycemia significantly (\*p<0.05, n≥11). Claudin-7 in hyperglycemia treated endothelial cell also decreased at (D) *cldn-7* mRNA expression (\*p<0.05, n=4) as well as (E) CLDN-7 protein expression (\*p<0.05, n=3). (F) Lower CLDN-7 was localized on cell membrane in hyperglycemia. CLDN-7(red) and PECAM-1(green, endothelial marker) are co-staining in HDMEC. (G) However, ZO-1 (green) aligned underneath of cell membrane was not changed by HG treatment. As same as mouse hearts, (H) *cldn-7* mRNA level decreased in diabetic heart induced by STZ (n=5, \*p<0.05), (J) but no significant different was shown in *zo-1* mRNA level (n=4). Statistical analysis performed using student’s t-test. (I) And lower CLDN-7(red) surrounded by alpha-SMA (green) was observed in heart tissue sections from STZD, (K) although there was no difference of ZO-1. Immunostaining for ZO-1 (green) to identify endothelial cell-specific tight junction structure, α-SMA (red) to identify vascular smooth muscle cells, and desmin (blue) to identify cardiomyocytes. Shown are representative images of n=3 mice per experimental group. High magnification images show junction formation between endothelial cells in small vessels. Scale bars: 50 μm and 150 μm, respectively.

**Figure 4. Reduced CLDN-7 expression decreased TEER and increased LMW of adiponectin movement across monolayer of HDMECs.** (A) One of major component of tight junction proteins, CLDN-7, was targeted to knock down by shRNA transfection after 96h. Transfected HDMECs were transferred into transmembrane culturing system (0.9cm2) and formed monolayer 72h after transfection. The cells having lower CLDN-7 expression (\*\*p<0.01, n=3) showed (B) significantly lower TEER (\*p<0.05, n≥7). TEER was measured at 96hr later from transfection starting. (C) The monolayer of CLDN-7 knockdown HDMECs were used for adiponectin permeability assay for 24hr. The decreased CLDN-7 led adiponectin movement increase, especially in LMW form with statistical significance (\*p<0.05, n≥5). The values were mean ± SEM and statistical analysis performed using student’s t-test.

**Figure 5.** **Functional 3D microvascular networks demonstrated size-dependent permeability and effect of hyperglycemia on adiponectin transport.** (A) Schematic representation of 3D perfusable microvascular network model with HUVECs in a PDMS (polydimethylsiloxane) microfluidic device. The PDMS mold has a central gel channel containing HUVEC cells and fibrin hydrogel bordered by triangle posts. The HUVEC/fibrin gel channel is flanked on both sides by fluid channels leading to four reservoirs filled with culture medium. (B) A device photo is shown with the area dimension (20,000 μm x 38,000 μm) and a dime as size reference. (C) Experiment timeline is shown. (D) Daily images (D1-D4) of HUVECs in the 3D fibrin gel shows cellular alignment dynamics during vasculogenesis (10X magnification was used). (E) The continuously GFP expressed HUVECs showed the perfusable vasculature on day 4. (F) Confocal reconstructed 3D image of VE-Cadherin (Red) stained with nucleus (DAPI, blue). A single plane from the stacked images showed three ortho-positions (x position/pink at 570 μm, y position/green at 700 μm and z position/blue at 18 μm) of continuous endothelial hollow. (G) To confirm the generated 3D vessels were perfusable, 5 μm beads conjugated with FITC were loaded into one reservoir and timelapes images were taken under fluorescence using Axio Observer Z1 microscope. (Time interval average was 1.4 sec.) (H) Rhodamine labeled full-length adiponectin (Rho-fAd, red) was injected with a concentration of 10 μg/mL. Fluorescent images were captured every 6-10 sec for 3 min from 2 or 3 different fields of view. Three representative images are shown at 0, 40, and 80 sec post infusion. To assess permeability, fluorescent signal intensity (AU) was quantified along a linear ROI spanning the microvessel-ECM interface as shown, and depicted as a distance from the ROI origin (closed circle). Permeabilized fluorescent signal were observed in the ECM (out of vessel) area overtime. Apparent permeability (Papp) was quantified using ImageJ with measurement from randomly selected ROIs. Hyperglycemia with 25mM glucose (J) did not change the permeability of 3kDa-FITC dextran. However, (I) 70kDa-Texas Red dextran and (K) Rho-fAd increased Papp significantly in high glucose (HG) treated condition. (D, E, G, H) The scale bar indicates 100 μm. (I-K) Data presented mean values ± SEM and error bars. Statistical evaluation was done by t-test with the values from 15-25 ROIs (4-6 different fields of view of 2-3 devices) for each condition. \* indicates p=0.0005 and \*\* indicates p<0.0001.

**Sup Figure 1. Full-length adiponectin (fAd) conjugated with VivoTag750 had 3 different forms by adiponectin oligomerization (LMW, MMW and HMW) and gave a linear slope of signal intensity depending on concentration of fAd.** (A) To confirm the labeled adiponectin, non-labeled fAd and VT750-conjugated fAd were separated side by side in a SDS-PAGE gel and scanned by Li-Cor image scanner. Then, the both separated fAds were transferred from the SDS-PAGE gel into PVDF membrane to be detected by adiponectin antibody via immunoblotting. It was confirmed that the labeled fAd had 3 different forms as same as non-labeled fAd. (HMW >250 kDa, MMW ~180 kDa and LMW ~90 kDa) (B) To assess the meaning of signal intensity with labeled adiponectin from the field of view under FMT system, fluorochrome concentration was measured in dose dependent conditions. It showed linear regression with R2=0.991 of the trendline. (C) FMT imaging was taken using a pin-channel imaging block and VT750-conjugated fAd was diluted serially in the range of 0 to 160 µg.

**Sup Figure 2. Equal amount of VT750-conjugated fAd was infused into mice via jugular vein cannula.** Serum samples were collected before adiponectin infusion (0min) as well as right after within 2 min. (A) The serum samples were separated in SDS-PAGE gel and scanned it with LiCor imager. 3 forms of labeled adiponectin were only detected from 2min samples with no different level of fluorescence intensity from control vs STZD animals. (B) Before labeled-adiponectin infusion, basal level of circulatory adiponectin amount was compared from control and diabetic, 4 days post induced from 150μg/g STZ injection by i.p., individuals (n>3). Serum level of adiponectin was measured by ELISA. At the same time, the total adiponectin level from the serum showed no changes in STZD mice comparing to control after labeled adiponectin infusion (at 2min).

**Sup Figure 3. FMT 2500 system could monitor biodistribution of VT750 over time in a mouse.** To validate feasibility of biodistribution monitoring with NIR probe, 2 nmol of protein free VT750 only was infused into mouse via tail vein and imaging with FMT was taken at 0, 10, 30, 60, and 90 min. The pattern of VT750’s circulation and accumulation was observed.

**Sup Figure 4. Hyperglycemia did not change adiponectin receptors.** In mouse hearts, STZ-induced hyperglycemina did not change AdipoR1 and AdipoR2 (A) mRNA and (B) protein expression. **(C)** As same as HDMEC, expression of adiponectin receptors (AdipoR1 and AdipoR2) were not changed in between hyperglycemia and control. (n=4)

**Sup Figure 5. Vasculature of HUVECs on a chip.** VE-Cadherin(red) was stained with DAPI(blue) after 96hr cell culture (at D4) with 50 ng/mL VEGF. During 4 days vasculature formation in hyperglycemia, the integrity of VE-C did not change when it compared to control in 2D endothelial monolayer of HUVECs as well as 3D perfusable vessels of HUVECs. All scale bars indicate 200 μm.

**Sup Video 1. 3D reconstruction of FMT/CT co-registration for control mouse.** Vehicle only infused animal was used. FMT data from Infused VT-750 labeled fAd indicated in color, and computed tomography (CT) data shown in white/grey.

**Sup Video 2. 3D reconstruction of FMT/CT co-registration for STZD mouse.** Rotated movie showed higher intensity of VT-750 labeled fAd (color) than control animal from FMT data. White/grey scale indicates CT data.

**Sup Video 3. 3D perfusable vasculature of HUVECs.** The confocal reconstruction showed 3D microvasculature with VE-Cadherin (Red) and nucleus (DAPI, blue) staining, which has a perfusable lumen aligned with interconnected endothelial cells.

**Sup Video 4. Perfusion of 5μm beads conjugated with FITC in microvascular network.** FITC polystyrene particles flow through the continuous 3D microvasculature.