Material and methods

Materials. Streptozotocin, vitronectin, and fluorescein-isothiocyanate (FITC) conjugated UEA-1 lectin from *Bandeiraea simplicifolia* were obtained from Sigma. (St. Louis, MO). Acetylated low-density lipoprotein (ac-LDL) from human plasma Dil complex (Dil-ac-LDL), and dihydroethidine (DHE) were purchased from Molecular Probes (Carlsbad, CA). Endothelial basal medium-2 (EBM-2) and its supplements (EGM-2 Bulletkit) were purchased from Lonza Inc. (Allendale, NJ). Matrigel, vascular endothelial growth factor-1 (VEGF-1), and mouse monoclonal anti-TSP-2 antibody were obtained from BD Biosciences (San Jose, CA). All other reagents were used at the highest available purity.

Animals. All animal procedures were performed according to the guidelines of the University of Pittsburgh and Michigan State University Institutional Animal Care and Use Committee (IACUC). The db/db mouse is an established model to study angiogenesis and vascular dysfunction in type 2 diabetes [1, 2]. Mice used in this study were adult male diabetic (db/db, BKS.Cg-m-/- Lepdb/Bom Tac) and non-diabetic healthy heterozygotes (db/+, BKS.Cg-m-/- Lepdb/_ lean), purchased from Jackson Labs (10-13 weeks old). Criteria for inclusion was blood glucose <200 mg/dL (normal, db/+) and blood glucose >300 mg/dL (type 2 diabetic, db/db). Type 1 diabetes was induced on male C57BL/6 mice (8-12 weeks, Jackson Laboratories) by intrapeeritoneal injection with streptozotocin (STZ; 45 mg/kg in sterile citrate buffer, for 5 consecutive days), as we previously described [3]. Mice with a blood glucose level > 280 mg/dL were considered diabetic [3, 4]. Blood glucose level and body weight were checked before animal sacrifice for both type 1 or 2 diabetes and their corresponding controls.

Isolation and characterization of bone marrow angiogenic cells (BMACs). *B*one marrow-derived angiogenic cells were isolated, cultured and characterized according to our previously described technique [1, 3, 5, 6] and according to previous literatures [7, 8]. Uptake of Ac-Dil LDL and binding of UEA-1 lectin, detection of cell surface markers using flow cytometry and detection of functional molecules using western blot were performed on the 7th day after isolation. In UEA-1 lectin binding assay, L-fucose was used to test whether the binding of UEA-1 lectin can be blocked by its hapten sugar (fucose). A 3D collagen cell culture system (Millipore) was used to test the in vitro tubulogenesis of these cells according to methods previously described [9]. BMACs were used for experiments on the 7th day after isolation.

In vitro tube formation assay. The tube formation capacity of BMACs was determined using Matrigel [1, 3, 5, 6]. In brief, 150 μl of Matrigel was solidified in 48-well plates, and BMACs were added to Matrigel at 5×10^4 cells/well. After 6 hrs of incubation at 37°C, the tubes were observed under an inverted microscope (Nikon). The tube number and tube length were analyzed in 4 random microscopic fields by Image-Pro Plus (Media Cybernetics Inc.).

Reactive oxygen species (ROS) measurement. The Intracellular BMAC ROS levels were evaluated using dihydroethidium (DHE) by flow cytometry or in fluorescence microscope [1, 6]. For flow cytometric analysis, BMACs were detached by trypsinization and incubated with DHE (1 μM, Molecular Probes) for 30 min at 37ºC in dark. After being washed with PBS, the cells were measured in FACScan (Becton Dickenson), and analyzed by CellQuest Software.

In the fluorescence microscopic analysis, BMACs were grown in LapTek II slide chamber (Nunc, Rochester, NY), and stained with DHE (1 μM). After DHE staining, cells were fixed with 2% PFA, and counterstained with DAPI. ROS level was determined by the ratio of the number of double positive cells for DHE and DAPI, normalized by the number of total cells positive for DAPI [10].

NADPH oxidase activity. NADPH oxidase activity was determined by a lucigenin-enhanced chemiluminescence [11, 12]. BMACs were lysed with a 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, and protease inhibitor cocktail (Sigma). The lysate was centrifuged at 12,000*g* for 30 min at 4°C,

and the supernatant was subjected to NADPH oxidase activity assay. The enzyme activity was measured by lucigenin (5 μM)-enhanced chemiluminescence in the presence of NADPH (100 μM). NADPH oxidase activity was calculated as relative light units (RLU)/μg protein [11].

Western blot. For the secreted protein, conditioned media were collected and concentrated using Amicon-Ultra centrifugation tubes (10 kDa cutoff, Millipore) as we previously described [6]. For cellular proteins, BMACs were lysed with CelLytic MT lysis buffer (Sigma) containing protease inhibitor cocktail (Sigma). The protein amounts in cell lysate or concentrated media were determined by BCA protein assay kit (Thermo Scientific). 300 μg/lane for media protein and 30 μg/lane for cellular protein were used for standard SDS-PAGE. Western blots were performed by using primary antibodies directed against thombospondin-2 (200 kDa TSP-2; 1:500, BD Biosceinces), Rac1 (21 kDa, 1:1,000, Abcam), gp91^{phox} (58 kDa, 1:1,000, BD Biosciences), p22^{phox} (22 kDa, 1:500, Santa Cruz), p47^{phox} (47 kDa, 1:500, Santa Cruz) and *β*-actin (42 kDa, 1:10,000, Sigma). Secondary antibody was IRDye 800 anti-mouse antibody (1:5,000, Rockland Immunochemicals), or Alexa Fluor 680 anti-rabbit antibody (1:2,500, Invitrogen). Bands were visualized with an Odyssey Imager (LI-COR bioscience) and quantified with Quantity One software (Bio-Rad).

In vitro gene transfer. The replication-incompetent adenoviral vectors were prepared as previously described [1, 13]. On the 6th day of cultivation, BMACs were infected with adenoviral vector encoding dominant-negative Rac1 (DN Rac1) or manganese superoxide dismutase (MnSOD) at a titer of 500 multiplicity of infection (MOI) in EGM-2 supplemented with 2% FBS. Adenovirus encoding β-galactosidase (β-gal) was used as treatment control. After 24 hrs of infection, media were changed with fresh EGM-2 containing 5% FBS, and cells were grown for an additional 48 hrs before conducting other experiments [14].

Quantitative real time PCR (qRT-PCR). Total RNA was extracted from cultured BMACs using RNeasy mini kit (Qiagen), and synthesized cDNA using TaqMan reverse transcription kit (Applied Biosystems). The specific primer sequences were as follows: 18S, forward: ACCGCAGCTAGGAATAATGGA; reverse: GCCTCAGTTCCGAAAACCA; thrombospondin-1 (TSP-1), forward: TCAGCTGTTAGGAACCTGAGGC; reverse: ACTGGTGAAGGGCCAAGATCT; and TSP-2, forward: TGAGTTCCAGGGCACACCA; reverse: GGCTTTCTGGGCAATGGTA. Quantification of gene copies was carried on the 7500 Real-Time PCR system, using Power SYBR Green master mix (Applied Biosystems). PCR cycles consisted of 3 stages with an initial step at 95ºC for 10 min followed by 40 cycles at 95ºC for 15 sec and 60 ºC for 1 min, and final stage for dissociation curve. Relative mRNA expressions were calculated by the comparative C_T method ($2^{-\Delta\Delta Ct}$), normalized to the endogenous 18S control.

Migration assay. To investigate BMAC migration activity, a modified Boyden chamber assay was performed using a transwell membrane (8 μm; Costar) . Described briefly, BM-BMACs were detached by trypsinization, harvested by centrifugation, and then resuspended with EBM-2 supplemented with 5% FBS. 5×10^4 BMACs were added to the upper chamber, and then the upper chamber was placed into the lower chamber filled with EGM-2 containing 5% FBS and human recombinant VEGF (50 ng/mL). After 24 hrs of incubation at 37°C, the upper side of the filter was gently scraped with cotton swabs to remove non-migrating cells. After being stained with Hoechst33528 (5 μM, Molecular Probes), cells migrating into the lower chamber were determined by counting the stained nuclei seen in fluorescence microscope (Nikon) with MetaMorph 6.1 software. Each experiment was performed in duplicate, and 3 random fields per filter were analyzed.

Adhesion assay. After detached by trypsinization, 5×10^4 BMACs/well were plated into 96-well plates. After 1 hr incubation at 37°C, non-adherent cells were rinsed away with PBS, and adherent cells were fixed with 2% PFA. Nuclei were stained with Hoechst33528 (5 μM) for 20 min at room temperature in the dark [15]. The

number of adherent cells was determined by counting the stained nuclei seen in fluorescence microscope (Nikon). Each experiment was performed in triplicate.

Isolation and measurement of microRNA let-7f. Enriched miRNAs were isolated from BMACs with the mirVana miRNA Isolation Kit (Ambion) as previously described [5]. qRT-PCR was performed with the mirVana qRT-PCR miRNA Detection Kit (Ambion). The U6 small nucleolar (sn) RNA was used as the housekeeping small RNA reference gene. The relative gene expression was normalized to U6 snRNA. Each reaction was performed in triplicate, and analysis was performed as described above in qRT-PCR. Primer identification numbers (Applied Biosystems) were as follows: has-let-7f, 000382; U6, 001973.

Transfection of let-7f mimic or let-7f inhibitor. For overexpression of let-7f, cells were transfected with 100 nM of miRIDIAN let-7f mimic or scramble miRNA mimic (Dharmacon), using DharmaFECT Transfection Reagent I (Dharmacon) according to the manufacturer's protocol. To inhibit the function of endogenous let-7f, miRIDIAN let-7f inhibitor or negative control oligonucleotides (Dharmacon) was transfected into normal BMACs. After 72 h of transfection, cells were harvested for further analysis.

Luciferase Target Assay of let-7f to 3'UTR of TSP-2 mRNA. The luciferase target assay was performed as previously described [16, 17]. Synthetic oligonucleotides as indicated by NCBI reference sequence bearing either 3'UTR clone of human TSP-2 (THBS2) mRNA 3'UTR(NM_003247.2), was cloned into pMirTarget plasmid (Origene, structure shown in Fig 7G) after the stop codon of luciferase, respectively. HEK 293 cells were co-transfected with 100 ng of TSP-2 (THBS2) 3'UTR plasmid and 0.1 nM of either let-7f mimic (Dharmacon) or negative control oligonucleotides (Dharmacon), all combined with Turbofect 8.0 (Origene) according to manufacturer's protocol. After 48 h, cells were washed and lysed with Reporter Lysis Buffer (Promega), and their luciferase activity was measured using the GloMax® 96 Microplate Luminometer (Promega). The relative reporter activity was obtained by normalization to each report plasmid and scramble oligo co-transfection. A reduced firefly luciferase expression indicates the direct binding of let-7f to TSP-2 mRNA 3'UTR.

Statistics. All obtained values were expressed as mean ± SEM. Statistical analysis between groups was performed using the Student's two-tailed t-test or one-way ANOVA followed by Duncan's test to determine the significant differences between treatment groups. Statistical analysis was performed using SPSS software version 17.01 (Chicago, IL). In all cases, a p value of < 0.05 was considered significant.

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