Oxidative Stress–Mediated Thrombospondin-2 Upregulation Impairs Bone Marrow–Derived Angiogenic Cell Function in Diabetes Mellitus

Ok-Nam Bae,* Jie-Mei Wang,* Seung-Hoon Baek, Qingde Wang, Hong Yuan, Alex F. Chen

Objective—Circulating angiogenic cells play an essential role in angiogenesis but are dysfunctional in diabetes mellitus characterized by excessive oxidative stress. We hypothesize that oxidative stress–mediated upregulation of thrombospondin-2 (TSP-2), a potent antiangiogenic protein, contributes to diabetic bone marrow–derived angiogenic cell (BMAC) dysfunction.

Approach and Results—BMACs were isolated from adult male type 2 diabetic db/db mice and control db/+ (C57BLKS/J) mice. In Matrigel tube formation assay, angiogenic function was impaired in diabetic BMACs, accompanied by increased oxidative stress and nicotinamide adenine dinucleotide phosphate oxidase activity. BMAC angiogenic function was restored by overexpression of dominant negative Rac1 or by overexpression of manganese superoxide dismutase. TSP-2 mRNA and protein were both significantly upregulated in diabetic BMACs, mediated by increased oxidative stress as shown by a decrease in TSP-2 level after overexpression of dominant negative Rac1 or manganese superoxide dismutase. Silencing TSP-2 by its small interfering RNA in diabetic BMACs improved BMAC function in tube formation, adhesion, and migration assays. Notably, the upregulation of TSP-2 was also found in BMACs from streptozotocin-induced type 1 diabetic mice, and normal BMACs with high glucose treatment. let-7f, a microRNA which has been related to endothelial angiogenic function, is found to play key role in TSP-2 increase, but let-7f did not directly interact with TSP-2 mRNA.

Conclusions—The upregulation of TSP-2 mediated by increased oxidative stress contributes to angiogenesis dysfunction in diabetic BMACs. (Arterioscler Thromb Vasc Biol. 2013;33:1920-1927.)

Key Words: angiogenesis inhibitors ■ diabetes ■ oxidative stress ■ stem cells ■ thrombospondin-2, human

Because ≤80% of all deaths in patients with diabetes mellitus are related to cardiovascular complications,1,2 there have been many attempts to clarify the cellular and molecular mechanisms for these cardiovascular symptoms in diabetes mellitus.3,4 Aberrant angiogenesis is one of the most serious symptoms associated with diabetes mellitus.5,6 Endothelial progenitor cells (EPCs) could be recruited from bone marrow to the injury site by homing signals to promote endothelial regeneration and neovascularization,7-9 suggesting the potential use of EPCs as clinical therapeutics to improve angiogenesis. However, the number of recruited EPCs and their function are decreased under diabetic conditions, implying that impaired EPC function could be critical for defective angiogenesis in diabetes mellitus.10-12 Although several studies have given some explanations for diabetic EPC dysfunction, including increased oxidative stress, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, and altered nitric oxide pathway,13,14 the mechanism underlying the defective angiogenic properties of diabetic EPCs remains largely unknown.

Thrombospondins (TSPs) consist of a family of extracellular glycoproteins, modulating cell-to-cell or cell-to-matrix communication, such as cell adhesion, proliferation, and migration.15,16 In their family of 5 members, TSP-1 and TSP-2 are known to form a subgroup and possess unique antiangiogenic characteristics.17 Although the antiangiogenic property of TSP-1 is well established in various cell systems,18,19 the antiangiogenic function of TSP-2 has not been extensively investigated. Interestingly, evidence from the clinical observations and our previous study demonstrate that oxidative stress is highly increased in EPCs from subjects with diabetes mellitus.12,20 Lopes et al21 showed that TSP-2 expression could be controlled by oxidative stress in endothelial cells. Until now, there have been no studies on the role of TSP-2

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in EPC function in either normal or disease states, including diabetes mellitus.

MicroRNAs (miRNAs) are small single-stranded RNAs that negatively regulate gene expression by binding to target messenger RNAs. In the fields of angiogenesis, it is reported that inhibitor against let-7f reduces endothelial sprout formation. However, the regulatory role of let-7f in angiogenesis is not completely understood yet.

Our laboratory has been working with EPCs in cardiovascular diseases, including diabetes mellitus, In recent years, the knowledge on EPC characterization has evolved. Several important phenotypes have been studied, including (1) expression of both stem cell markers and endothelial cell markers; (2) expression of endothelial cell functional genes; (3) uptake of acetylated low-density lipoprotein and lectin binding; (4) production of proangiogenic factors that facilitate vascular formation; (5) incorporation into the vasculature or formation of tubular structure with lumen, etc.

In 3-dimensional (3D) collagen gel, these cells are capable of forming tubular structures (Figure 1C). Our flow cytometry analysis also indicated that 7-day cells displayed higher expression of stem cell markers and endothelial cell makers than freshly isolated cells (Table). These cells are heterogeneous, and small fraction of these cells have possibility of being endothelial cells on the basis of CD144 expression. In 3-dimensional (3D) collagen gel, these cells are capable of forming tubular structures (Figure 1C). To compare the angiogenesis capacity between BMACs isolated from normal (db/+ and type 2 diabetic (db/db) mice, BMACs were plated onto Matrigel and their ability to form tubes and networks was evaluated. BMACs from diabetic mice had significantly impaired tube formation ability, as shown by both tube number and tube length (Figure 1D).

**Impaired Angiogenesis in Diabetic BMAC Is Restored by Inhibition of NADPH Oxidase**

To investigate the mechanism underlying diabetic BMAC dysfunction, we measured the extent of oxidative stress using dihydroethidium. As shown in Figure 2, the dihydroethidium signal in diabetic BMACs was significantly increased compared with normal BMACs, in both flow cytometric (Figure 2A) and fluorescence microscopic (Figure 2B) analysis. To clarify how oxidative stress is increased in diabetic BMACs, we measured NADPH oxidase activity, which is known as one of the enzyme systems most responsible for reactive oxygen generation in vascular tissue, using lucigenin-enhanced chemiluminescence. NADPH oxidase activity was highly increased in diabetic BMACs (Figure 2C), and the protein levels of p47phox, gp91phox, and Rac 1, important NADPH oxidase subunits, were significantly enhanced in diabetic BMACs (Figure 2D). To further investigate the possible contribution of increased NADPH oxidase activity, we transfected adenoviral vector–mediated dominant negative Rac1 (DN Rac1), which can retard the function of endogenous Rac1 or its control vector expresses β-galactosidase. Overexpression of DN Rac1 decreased NADPH oxidase activity in diabetic BMACs (Figure 2E, left) and significantly restored impaired ability for tube formation (Figure 2F, right).

**Antiangiogenic Protein TSP-2 Is Significantly Upregulated in Diabetic BMACs**

To identify the target protein of oxidative stress in impaired diabetic BMAC angiogenesis, we examined antiangiogenic TSP-2 levels, on the basis of a previous study showing that TSP-2 could be regulated by oxidative stress in normal endothelial cells. Real-time PCR results revealed that TSP-2 mRNA was highly increased in diabetic BMACs, whereas TSP-1 was not changed (Figure 3A). The increased TSP-2 in diabetic BMACs was also found by immunostaining (Figure 3B). Usually, TSP-2 is known to be synthesized and secreted to extracellular media to exert its function in cell–cell or cell–matrix interaction. In Western blot analysis, newly synthesized cytosolic form in diabetic BMACs was increased (Figure 3C, left), which is well correlated with mRNA level. The main functional form of secreted TSP-2 was also significantly increased, as analyzed in conditioned media after diabetic BMAC incubation (Figure 3C, right).

**TSP-2 Upregulation in Diabetic BMACs Is Mediated by Increased Oxidative Stress in Diabetic BMACs**

To examine the relationship between increased oxidative stress and TSP-2 upregulation in diabetic BMACs, we determined TSP-2 level after modulating oxidative stress using the adenoviral vector–overexpressing dominant negative Rac1 interferes with endogenous Rac1, the major component of NADPH, hence the NADPH activity was significantly decreased after adenovirus-expressing dominant negative Rac1 transfection in db/db BMACs (Figure 2D). MnSOD is the main antioxidant enzyme found in BMACs.
Adenovirus-overexpressing MnSOD is expected to decrease oxidative stress in cells. As shown in Figure 4A, increased oxidative stress in diabetic BMACs was reversed after overexpression of DN Rac1 or MnSOD. Upregulation of TSP-2 mRNA and protein level was significantly diminished after inhibition of oxidative stress (Figure 4B and 4C), demonstrating that increased oxidative stress was the main cause for the upregulation of TSP-2 in diabetic BMACs.

Upregulated TSP-2 Plays a Key Role in Impairment of Diabetic BMAC Function
To determine the role of increased TSP-2 in diabetic BMAC dysfunction, we introduced silencing RNA against TSP-2 to diabetic BMACs. The viability and morphology of BMACs were not affected (data not shown). The protein level of TSP-2 was significantly decreased after 72 hours of transfection (Figure 5A). In Matrigel tube formation assay, silencing RNA against TSP-2 restored the impaired BMAC function, as shown by an increase in tube number and tube length (Figure 5B). Besides tube formation ability, adhesion and migration ability, which are the key features of BMAC angiogenic function, were significantly impaired in diabetic BMACs, but were remarkably improved by inhibition of TSP-2 using its small interfering RNA (Figure 5C and 5D).

Expression of TSP-2 Is Increased in BMACs Under High Glucose Conditions or in BMACs Isolated From Type 1 Diabetic Mice
To further explore the relevance of TSP-2 upregulation in diabetic BMACs, we used 2 representative diabetic models. We simulated high glucose conditions in patients with diabetes mellitus with an in vitro model, by incubating normal BMACs in normal (5 mmol/L glucose and 25 mmol/L mannitol) and high glucose media (30 mmol/L glucose) for 7 days.30 High glucose itself increased cellular oxidative stress in normal BMACs (Figure 6A). Notably, TSP-2 level was also significantly upregulated (Figure 6B). A similar increase in TSP-2 level was observed in BMACs isolated from streptozotocin-induced type 1 diabetic mice (Figure 6C). These data suggest that the upregulation of TSP-2 can be observed throughout the different types of diabetes mellitus.
MiRNA let-7f Contributes to TSP-2 Upregulation and Dysfunction in Diabetic BMACs

To investigate whether TSP-2 upregulation and impaired function of diabetic BMACs are under regulation of miRNA, we examined the level of let-7f, a representative miRNA related to angiogenic function in endothelial cells. The level of let-7f is significantly decreased in diabetic BMACs (Figure 7A). When let-7f was overexpressed by transfection of let-7f mimic (Figure 7B), impaired tube formation of diabetic BMACs was rescued (Figure 7C) and the increased protein level of TSP-2 was reduced to the normal level (Figure 7D). Notably, the restoration of upregulated TSP-2 level by let-7f was also observed in BMACs isolated from type 1 diabetic animals (Figure 7E), showing that TSP-2 was regulated by let-7f in diabetic BMACs. Consistently, the impaired angiogenic function and the increased level of TSP-2 were observed in normal BMACs after inhibition of endogenous let-7f by transfection with let-7f inhibitor oligonucleotides (Figure 7F–7H). However, let-7f does not directly interact with the 3′-untranslated region of TSP-2 mRNA, as found in the luciferase assay using reporter vector inserted with 3′-untranslated region of human TSP-2 (THBS2) mRNA (Figure 7I).

Discussion

In the present study, we demonstrated that increased NADPH oxidase activity and resultant oxidative stress contribute to diabetic BMAC dysfunction, mediated by upregulation of the antiangiogenic protein TSP-2. To our best knowledge, this is the first study showing that TSP-2 could play a key role in BMAC function. Downregulation of TSP-2 by its small interfering RNA significantly restored BMAC functions in tube formation, adhesion, and migration, indicating the critical roles of TSP-2 in angiogenic BMAC function. Furthermore, TSP-2 upregulation was also observed in BMACs under hyperglycemic conditions or isolated from type 1 diabetes mellitus, suggesting its potential contribution to BMAC dysfunction in patients with diabetes mellitus.

In this study, we have investigated the antiangiogenic role of TSP-2 in BMACs, which has been ignored compared with that of TSP-1. Despite the high degree of structural similarity between TSP-1 and TSP-2, it is reported that they are differently regulated on expression pattern, reflecting their distinct roles. The role of TSP-1 in the vascular impairment of the diabetic condition has been suggested by several studies using vascular tissues. Recently, Li et al. reported that TSP-1 might be important in impairment of EPC-associated neovascularization. They found that the expression of TSP-1 in diabetic EPCs was similar to that in db/m EPCs under low-glucose conditions, which is consistent with our results (Figure 3A). In this regard, our results showing that TSP-2 is highly upregulated in both types of diabetes mellitus and under hyperglycemic condition suggest that TSP-2 could be an important mediator of diabetic vascular impairment, raising the need for further investigation of the role of TSP-2 in diabetic vascular dysfunction.

Figure 2. Role of oxidative stress in diabetic bone marrow–derived angiogenic cell (BMAC) dysfunction. Reactive oxygen species generation was determined by flow cytometry (A) or fluorescence microscope (B) after dihydroethidium (DHE) staining (scale bar, 200 μm). (C) Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was detected in luminometer. (D) NADPH oxidase subunits were examined using Western blot. (E) Effects of adenovirus dominant negative Rac1 (Ad-DN Rac1) overexpression on NADPH oxidase activity. (F) Effects of dominant negative Rac1 (Ad-DN Rac1) overexpression on diabetic BMAC function (scale bar, 500 μm). Ad-DN galactosidase (β-Gal) was used as control (A, n=8; B, n=4; C, n=4; D, n=4 to 7; E, n=4; and F, n=5). *P<0.05 vs db/+; #P<0.05 vs db/db β-Gal. Data are expressed as mean±SEM and analyzed with Student t test.
tissues. Supporting our view, a recent study demonstrated that TSP-2 could play a critical antiangiogenic role in ECs, resulting in inhibition of physiological angiogenesis.33

Consistent with these present observations, increased oxidative stress has been reported to be the potential mechanism responsible for defective vascular function in patients with diabetes mellitus and animal models.20,34,35 Although here we used dihydroethidium, which may not be sufficient to specify between hydrogen peroxide and superoxide anions, many previous studies reported increased reactive oxygen species (ROS) generation under diabetic conditions. In vascular tissues isolated from patients with diabetes mellitus, NADPH oxidase activity is increased via upregulation of NADPH oxidase subunits, leading to enhanced generation of superoxide anion.39 In BMACs from patients with diabetes mellitus, NADPH oxidase activity is found to be increased,20,34 and superoxide generation is significantly increased.20 Supporting our view that increased oxidative stress plays an important role for diabetic EPC dysfunction, a recent review summarized that imbalanced ROS generation could contribute to EPC dysfunction under disease condition. Inhibition of oxidative stress has reversed diabetic EPC dysfunction in previous studies.36 Selective Rac1 inhibition in diabetic mice reversed increased oxidative stress, resulting in improvement of vascular function.37 Inhibition of NADPH oxidase subunit p47phox or scavenging oxidative stress could restore the EPC function,20 and MnSOD expression in diabetic EPC restored impairment of diabetic wound healing.12 Nevertheless, most studies have focused on the oxidative stress itself without demonstrating its possible downstream targets, such as antiangiogenic protein. In this regard, our study could provide a new insight into the role of increased oxidative stress in cellular signaling, including protein expression, under diabetic conditions.

Interestingly, Tang et al.38 recently demonstrated that retinal endothelial cells isolated from CYP1B1 knockout mice exhibited impaired migration and capillary morphogenesis by upregulation of TSP-2, which could be reversed by antioxidant, supporting our view that oxidative stress could be the key regulator for TSP-2 expression. Our data showed the involvement of oxidative stress in TSP-2 regulation with 2 different approaches, either by blocking ROS generation or by upregulating the endogenous antioxidant system (Figure 4).

Figure 3. Upregulation of thrombospondin-2 (TSP-2) in diabetic bone marrow–derived angiogenic cell (BMACs). A, TSP-1 or TSP-2 mRNA in normal and diabetic BMAC was measured by quantitative reverse transcriptase polymerase chain reaction. B, Expression of TSP-2 was determined in fluorescence microscope. C, Cytosolic (left) or secreted (right) TSP-2 protein level was detected in Western blot (A, n=3 to 6; B, n=3; and C, n=4 to 6; scale bar, 200 μm). *P<0.05; **P<0.01 vs db/+. Data are expressed as mean±SEM and analyzed using Student t test or 1-way ANOVA followed by Duncan test to determine the significant differences between treatment groups.

Figure 4. Regulation of thrombospondin-2 (TSP-2) by oxidative stress in diabetic bone marrow–derived angiogenic cells (BMACs). A, Effect of adenovirus dominant negative Rac1 (Ad-DN Rac1) or Ad-manganese superoxide dismutase (MnSOD) on oxidative stress in BMACs. Expression of TSP-2 was determined in mRNA level (B) and protein level (C) after modulation of oxidative stress (A, n=4; B, n=3 to 6; and C, n=4 to 8; scale bar, 200 μm; *P<0.05 vs db/+; #P<0.05 vs db/db β-galactosidase (β-Gal). Data are expressed as mean±SEM and analyzed with 1-way ANOVA followed by Duncan test to determine the significant differences between treatment groups.
To confirm the role of DN Rac1, we demonstrated that activity of NADPH oxidase or oxidative stress is reversed (Figures 2E and 4A), and both of them are positively correlated with TSP-2 mRNA/protein level (Figure 4B and 4C). To scavenge the ROS, we investigated MnSOD, on the basis of its unique contribution to BMAC antioxidant system. The expression of MnSOD, not CuZnSOD or catalase, is found to be 3- to 4-fold higher in BMAC than that in ECs, representing a critical mechanism for intrinsic resistance of BMACs against ROS. Decrease of TSP-2 mRNA/protein level after MnSOD overexpression is well correlated with the reverse of increased ROS by MnSOD (Figure 4), reflecting the importance of MnSOD in dysregulation of ROS in diabetic BMACs. Because of the lack of previous studies about the upstream pathway of TSP-2, the exact mechanism allowing oxidative stress to enhance TSP-2 expression has not yet been clarified. However, the observation that oxidative stress regulates the antiangiogenic protein of TSP-2 could give a possible explanation for BMAC impairment under high oxidative stress, as found in diverse cardiovascular disease, including diabetes mellitus, hypertension, coronary artery disease, or after smoking.

Here, we have used in vitro models of adhesion, migration, and tube formation, to evaluate BMAC functions under diabetes mellitus. Each of these assays represents the critical stages that occur during BMAC-mediated angiogenesis. Importantly, silencing TSP-2 could improve all of these parameters (Figure 5). These results suggest that TSPs could modulate diverse cellular interactions, including cell adhesion and migration, and are well supported by the literature. An issue of using Matrigel for tube formation is that it does not indicate tubular structure with lumens. Therefore, more comprehensive approaches are needed to fully represent the angiogenic potential of BMACs. In addition, it would be interesting to look into the regulation of TSP-2 in BMAC proliferation and apoptosis in future, which represent important functional aspects of BMACs. Considering the recent progress that BMACs could be novel targets for cell therapy with possible genetic modulation, further in vivo studies would be necessary to suggest that modulation of TSP-2 could be a possible therapeutic target for vascular dysfunction in diabetes mellitus.

In this study, we have investigated the possible contribution of miRNA dysregulation to angiogenic BMAC function. Disturbed level of miRNAs has been suggested to play a role in BMAC dysfunction under pathological condition. Consistent with a previous study showing that let-7f is involved...
in angiogenic function in endothelial cells, here we found that the level of miRNA let-7f was decreased under diabetic condition (Figure 7A). Notably, the reduced level of let-7f is a causative factor for TSP-2 upregulation, as well as functional impairment in both type 1 and type 2 diabetic BMACs (Figure 7B–7D). Meanwhile, let-7f does not directly interfere with TSP-2 translation, as shown in luciferase target assay (Figure 7E). Moreover, the potencies for recovery of tube formation ability of diabetic BMACs are different between silencing RNA against TSP-2 and let-7f mimic (Figures 5B and 7C), suggesting the existence of other regulatory pathways for TSP-2 upregulation in diabetic BMACs other than let-7f. Further investigation will be required to clarify how let-7f and regulating pathways modulate TSP-2 level in BMACs.

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Figure 7. Involvement of let-7f in thrombospondin-2 (TSP-2) upregulation and diabetic bone marrow–derived angiogenic cell (BMAC) dysfunction. A, The level of let-7f was measured from BMACs using quantitative reverse transcriptase polymerase chain reaction after isolation of miRNA. The U6 small nucleolar (sn) RNA was used as the housekeeping small RNA reference gene. The level of miRNA let-7f (B), tube formation ability (C), and the protein level of TSP-2 (D) were determined after overexpression of let-7f by transfection of let-7f mimic (let-7f m) to BMACs isolated from db/+ or db/db mice. Negative control oligonucleotides (Neg) were used as control. E, The effect of let-7f mimic on TSP-2 level in type I diabetic BMAC was determined. The level of miRNA let-7f (F), tube formation ability (G), and the protein level of TSP-2 (H) were determined after inhibition of let-7f by transfection of let-7f inhibitor (let-7f inh) to normal BMACs. I, Luciferase activity was measured after cotransfection of TSP-2 (THBS2) 3′-untranslated region plasmid with let-7f or negative control to human embryonic kidney 293 cells. The structure of luciferase reporter plasmid is shown in left (A, n=6; B, n=4; C, n=4; D, n=4; E, n=4 to 5; F, n=4; G, n=4; H, n=4; and I, n=3). *P<0.05; **P<0.01 vs db/+ or corresponding control; #P<0.05 vs Neg. Data are expressed as mean±SEM and analyzed using Student t test.

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Disclosures

None.

References


Significance
Oxidative stress plays an important role in the defective vascular function in various diseases, including diabetes mellitus. This study demonstrated that increased nicotinamide adenine dinucleotide phosphate oxidase activity induces upregulation of the antiangiogenic protein of thrombospondin-2, resulting in impairment of diabetic bone marrow–derived angiogenic cell functions. These findings provide useful information on oxidative stress–mediated modulation of angiogenesis. Considering recent progress that bone marrow–derived angiogenic cells could be candidate seed cells for therapy with possible genetic modulation, modulation of thrombospondin-2 could be a possible therapeutic target for vascular dysfunction in cardiovascular diseases.
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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. 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 intraperitoneal injection with streptozotocin (STZ; 45 mg/kg in sterile citrate buffer, for 5 consecutive days), as we previously described. Mice with a blood glucose level >280 mg/dL were considered diabetic. 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).** Bone marrow-derived angiogenic cells were isolated, cultured and characterized according to our previously described technique and according to previous literatures. 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. 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. 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. For flow cytometric analysis, BMACs were detached by trypsinization and incubated with DHE (1 µM, Molecular Probes) for 30 min at 37°C 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.

**NADPH oxidase activity.** NADPH oxidase activity was determined by a lucigenin-enhanced chemiluminescence. 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 12,000g 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.

**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. For cellular proteins, BMACs were lysed with CellLytic 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 thrombospondin-2 (200 kDa TSP-2; 1:500, BD Biosciences), Rac1 (21 kDa, 1:1,000, Abcam), gp91phox (58 kDa, 1:1,000, BD Biosciences), p22phox (22 kDa, 1:500, Santa Cruz), p47phox (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. 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.

**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: GGCTTCTGGGCAATGGTA. 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 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60 ºC for 1 min, and final stage for dissociation curve. Relative mRNA expressions were calculated by the comparative Ct method (2^(-∆∆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. 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. 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 miRDIAN 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, miRDIAN 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. 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.
References


