

Angiotensin II Downregulates MicroRNA-145 to Regulate Kruppel-like Factor 4 and Myocardin Expression in Human Coronary Arterial Smooth Muscle Cells under High Glucose Conditions

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SUPPLEMENTAL METHODS

Reactive Oxygen Species (ROS) Measurement of HCASMCs

For the measurement of ROS of HCASMCs, we used OxiSelect™ In Vitro ROS/RNS Assay Kit (Cell Biolabs, San Diego, CA) according to the manufacturer's protocol. The fluorescence was read with a fluorescence plate reader (GloMax, Promega, Madison, WI) at 490nm excitation /530nm emission.

Promoter Activity Assay

A-2134 to -1641 bp human *Klf4* promoter construct was generated as follows. Human genomic DNA was amplified with forward primer 5'-ACGCGTCTGTGGTCCGGCGA GGAGTGGAA-3' and reverse primer 5'-AGATCTCCACCGGGAAGGGA ATAGGAAAC-3'. The amplified product was digested with *MluI* and *BglII* restriction enzymes and ligated into pGL3-basic luciferase plasmid vector (Mission Biotech, Taipei, Taiwan) digested with the same enzymes. The *Klf4* promoter contains *miR-145* conserved sites (GGGGGGGGGG) at -1896 to -1892 bp and -1880 to -1875 bp. *MiR-145* binding site in the promoter of *Klf4* was identified by the software of Lasergene-MegAlign module (DNASTAR, WI, USA). For the mutant, the *miR-145* binding

sites were mutated using the mutagenesis kit (Stratagene, La Jolla, CA). Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into HCASMCs using a low pressure-accelerated gene gun (Bioware Technologies, Taipei, Taiwan) essentially following the protocol from the manufacturer. In brief, 2 µg of plasmid DNA was suspended in 5 µl of PBS and was delivered to the cultured cells at a helium pressure of 15 psi. The transfection efficiency using this method is 30%. Following 4 h of high glucose stimulation (25 mM), cell extracts were prepared using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and measured for dual luciferase activity by luminometer (Turner Designs, Sunnyvale, CA, USA). *Renilla* luciferase activity was normalized to firefly luciferase activity.

Streptozotocin (STZ) Animal Model

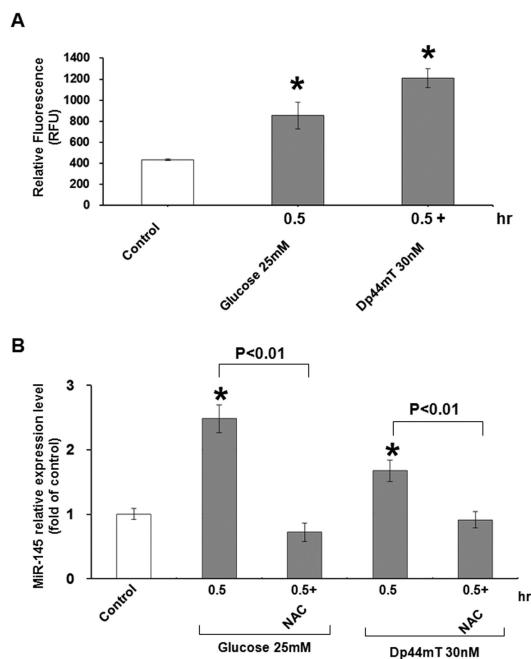
Male Wistar rats (220-250g), age 15 wks, which were kept under conventional conditions with free access to water and standard food. Experiment diabetes was induced in the rats by a single intraperitoneal injection of streptozotocin (STZ, Sigma, St Louis, MO, USA), dissolved fresh in sterile citrate buffer 0.01M, pH4.5, at a dose of 40 mg/kg body weight. Equal volumes

of this vehicle were injected into the control rats. At 7 d after induction, blood was collected by tail bleeding and assayed using the glucometer. Diabetes was confirmed by the presence of hyperglycaemia (19.4 mmol/l) for at least 1 wk. All animal procedures were performed in accordance with institutional guideline and conformed to *Guide for the Care and Use of Laboratory Animals* as adopted by the US National Institutes of Health.

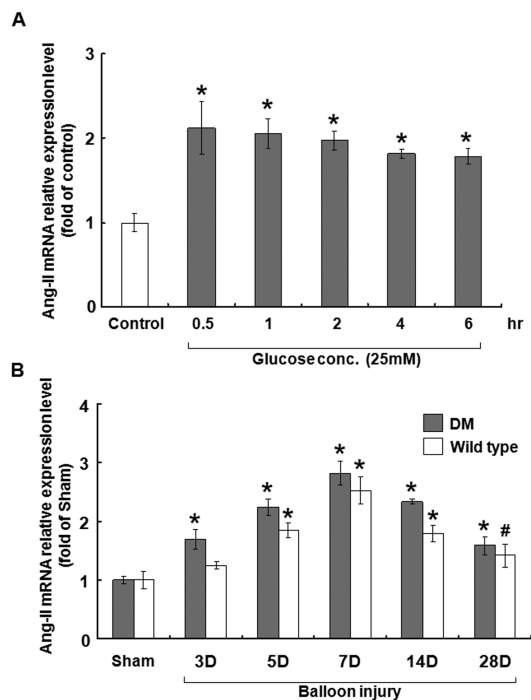
Balloon Injury of Rat Carotid Artery

Adult Wistar rats were anesthetized with isoflurane (3%) and subject to balloon catheter injury of the right carotid artery as described previously.¹² Briefly, a 2F Forgarty balloon catheter (Biosensors International Inc., Newport Beach, CA) was inserted through the right external carotid artery, inflated and passed 3 times along the length of the isolated segment (1.5 to 2 cm in length), then the catheter was removed. *miR-145* expression vector (pmR-ZsGreen1, Clontech) was injected to the segment, and electric pulses using CUY21- EDIT Square Wave Electroporator (Nepa Gene) were administered with five pulses and five opposite polarity pulses at 250 V/cm, 50 ms duration, 75 ms interval using Parallel fixed platinum electrode (CUY610P2-1, 1 mm tip, 2 mm gap). The injected *miR-145* ex-

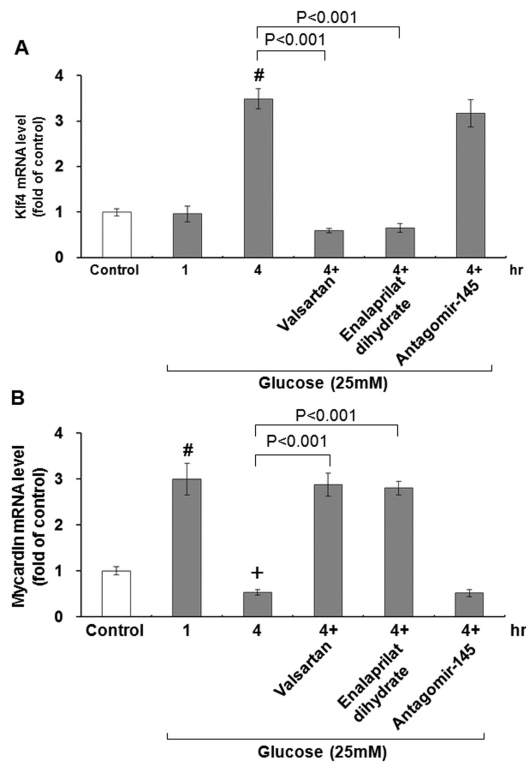
pression vector was incubated for 10 min. After incubation, unbound plasmid was aspirated. The carotid artery was then tied off, and the neck was closed. The rats were sacrificed at 3 to 28 d after balloon injury by placing rats in a close container and fill with CO₂ (60%). The CO₂ was used to fill of the chamber gradually to displace 10-30% of the chamber volume per minute. CO₂ flow was maintained at gas level of 60% for 2 min after respiratory arrest of the rats. Then the carotids were perfusion fixed at constant physiologic pressure with 4% paraformaldehyde. The vessels were excised and embedded in paraffin blocks, and two cross-sections were cut at positions 1 and 2 cm upstream of the carotid bifurcation. Intimal, medial, and adventitial cross-sectional areas were measured by a image software (Image Pro® Plus Olympus BX 51, Tokyo, Japan).



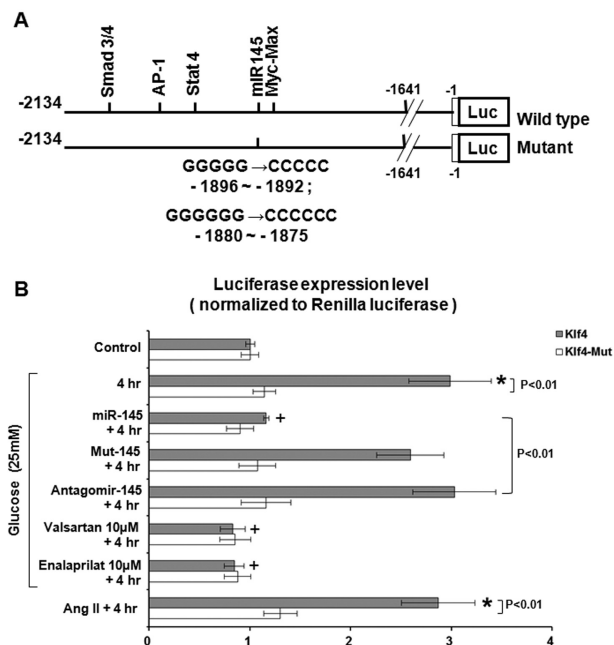
Supplementary Figure S1. Effect of high glucose on reactive oxygen species production and miR-145 expression in HCASMCs. (A) High glucose increased ROS production. *P<0.01 vs. control. N=3 per group. (B) High glucose and Dp44mT increased miR-145 expression and NAC attenuated the increased miR-145 by high glucose and Dp44mT. *P<0.01 vs. control. N=3 per group.



Supplementary Figure S2. Effect of high glucose stimulation on angiotensin II mRNA expression. Ang II mRNA was quantified by real-time PCR. (A) Quantitative analysis of Ang II mRNA expression in HCASMCs. *P<0.01 vs. control. (n=3). (B) Quantitative analysis of Ang II mRNA expression in arterial wall of balloon-injured carotid artery in diabetic and wild type rats. *P<0.01 vs sham. #P<0.05 vs. sham. (n=4).



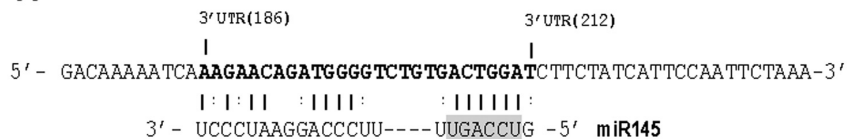
Supplementary Figure S3. Effect of valsartan, enalaprilat and antagomir145 on Klf4 and myocardin mRNA expression under high glucose stimulation. Pretreatment of valsartan and enalaprilat reversed Klf4 and myocardin mRNA expression induced by high glucose stimulation. [#]P<0.01 vs. control. ⁺P<0.05 vs. control. (n=4 per group).



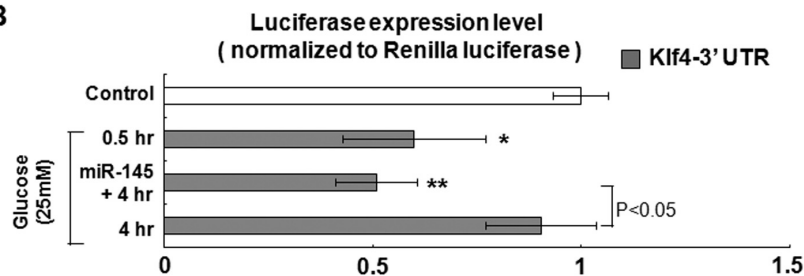
Supplementary Figure S4. High glucose increases *Klf4* promoter activity. (A) Constructs of wild type and mutant type of *Klf4* promoter. Mutant *Klf4* promoter indicates mutation of miR-145 binding sites in the *Klf4* promoter as indicated. (B) Quantitative analysis of *Klf4* promoter activity. Cultured HCASMCs were transiently transfected with pKlf4-Luc by gene gun. The luciferase activity in cell lysates was measured and was normalized with Renilla activity by Dual-Luciferase Reporter Assay System. ^{*}P<0.01 vs. control. ⁺<0.01 vs. high glucose 4 h. (n=3 per group).

MOLECULAR REGULATION OF miR-145 BY HIGH GLUCOSE

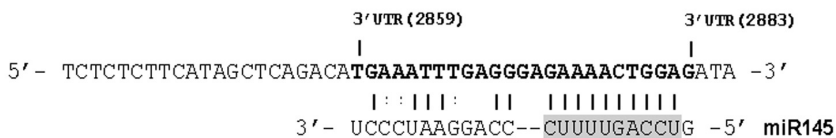
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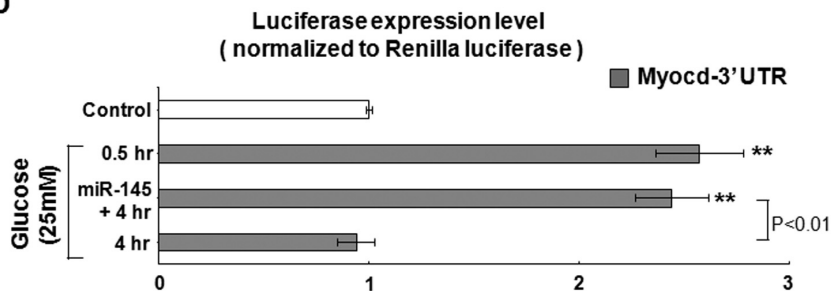
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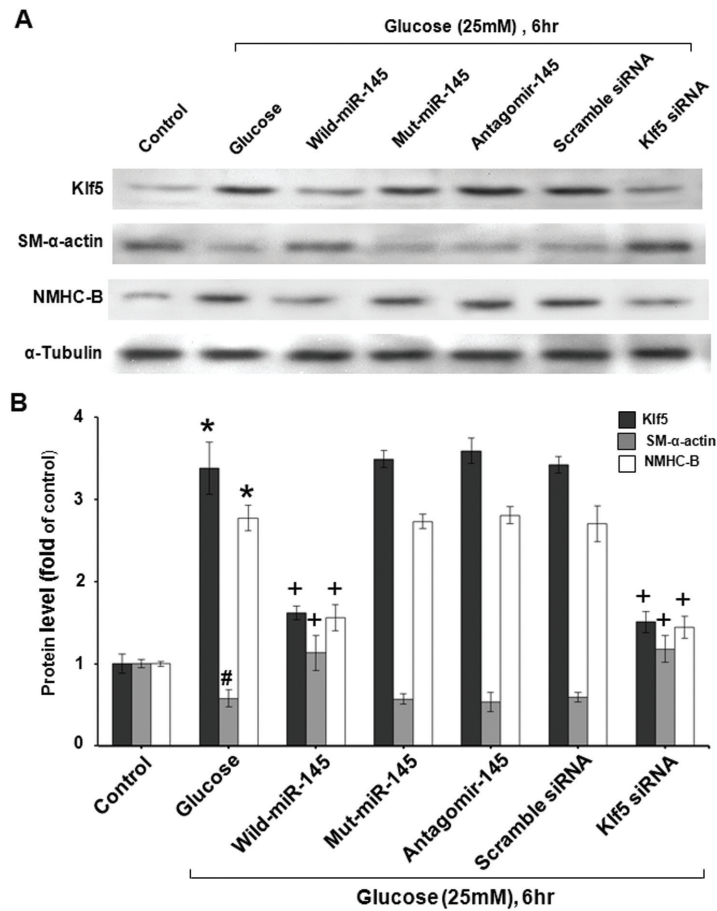
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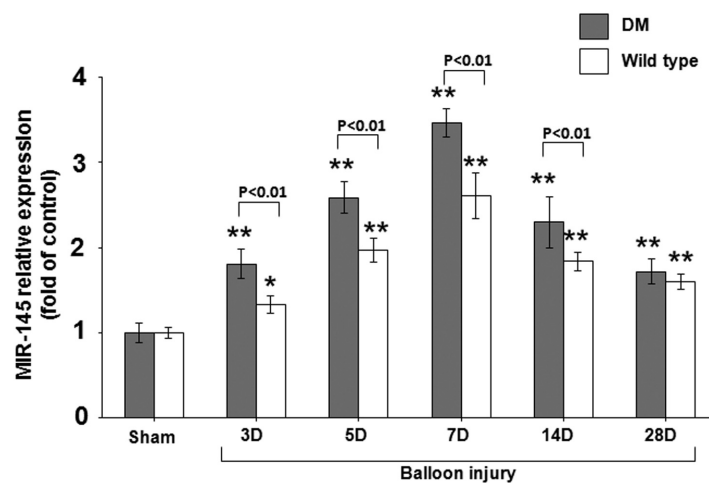
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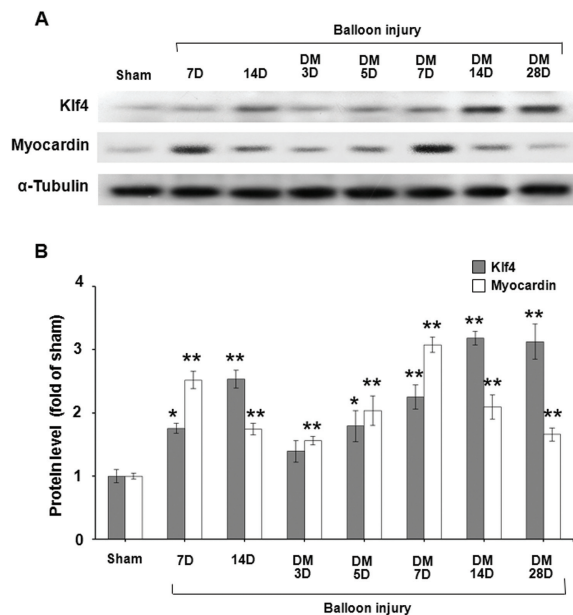
Supplementary Figure S5. Effect of high glucose and miR-145 on *Klf4* 3'-UTR and *myocardin* 3'-UTR by luciferase assay. (A and C) Construct of *Klf4* 3'-UTR and *myocardin* 3'-UTR. (B and D) Quantitative analysis of luciferase activity, miR-145 + 4 hr indicates overexpression of miR-145 in HCASMCs under glucose stimulation for 4 h. *P<0.05 vs. control. **P<0.01 vs. control. (n=3 per group).



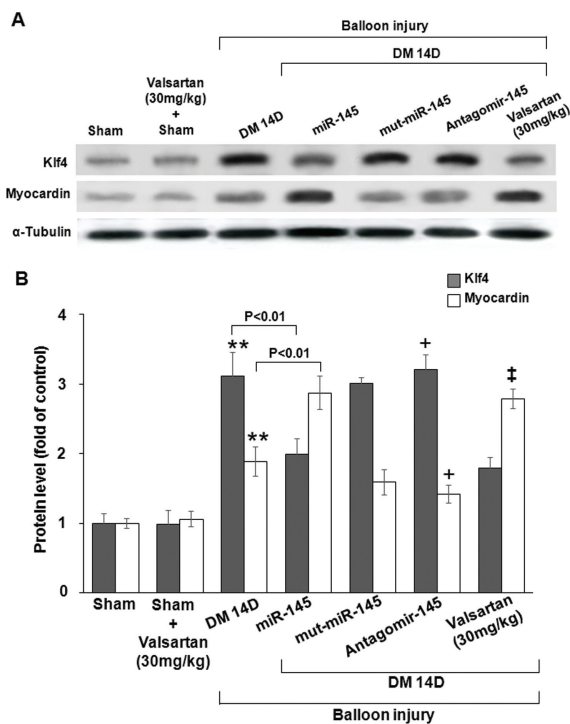
Supplementary Figure S6. Effect of high glucose stimulation on Klf5 protein expression and VSMC phenotype. (A) Representative western blots for Klf5, smooth muscle(SM)-α-actin, and nonmyosin heavy chain (NMHC)-B in HCASMCs after high glucose stimulation for 6 h in the presence or absence of overexpression of miR-145, mut-miR-145, antagomir-145, scramble-siRNA, and Klf5 siRNA. (B) Quantitative analysis of Klf5, smooth muscle(SM)-α-actin, and nonmyosin heavy chain (NMHC)-B protein levels. The values after high glucose stimulation have been normalized to matched α-tubulin measurement and then expressed as a ratio of normalized values to protein in control group. **P<0.01 vs. control. #P<0.05 vs. control. *P<0.01 vs. glucose. (n=3 per group).



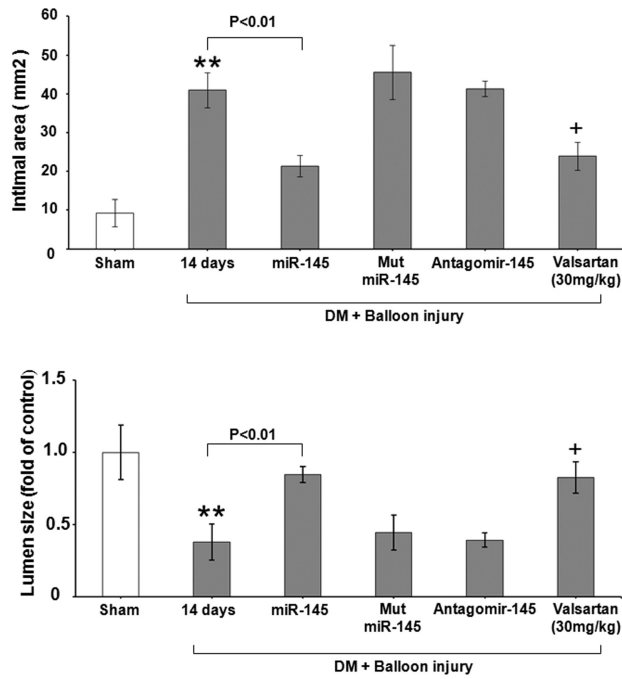
Supplementary Figure S7. Effect of balloon injury of carotid artery on miR-145 expression in diabetic and wild type rat. Carotid artery balloon injury was performed for 3 to 28 d. *P<0.05 vs. sham. ** P<0.01 vs. sham. (n=6 per group).



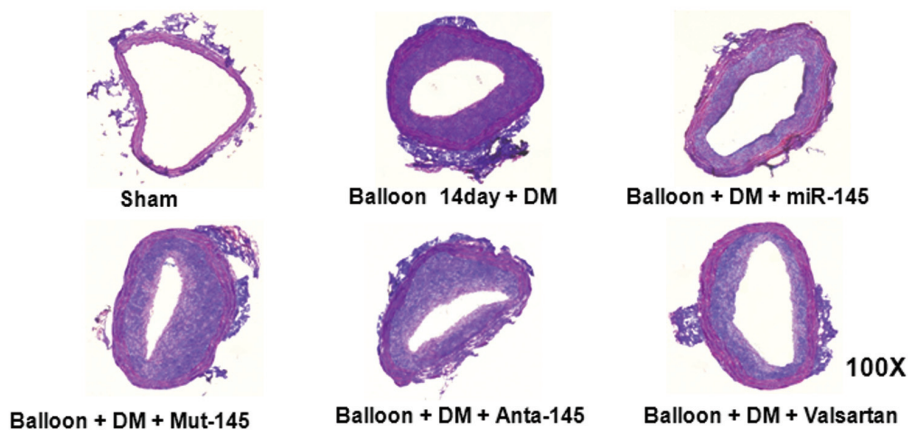
Supplementary Figure S8. Effect of carotid balloon injury on Klf4 and myocardin protein expression in diabetic and wild type rats. (A) Representative western blots for Klf4 and myocardin in arterial tissue after balloon injury for various periods of time. (B) Quantitative analysis of Klf4 and myocardin protein levels. The values after balloon injury have been normalized to matched α -tubulin measurement and then expressed as a ratio of normalized values to protein in the sham group. * $P < 0.05$ vs. sham. ** $P < 0.01$ vs. sham. (n=6 per group).



Supplementary Figure S9. Effect of miR-145 and valsartan on Klf4 and myocardin protein expression after carotid artery balloon injury in diabetic rats. (A) Representative Western blots for Klf4 and myocardin protein levels in carotid arterial tissue after balloon injury for 14 d in the absence or presence of wild type miR-145, antagomir-145, mutant miR-145, and valsartan. (B) Quantitative analysis of Klf4 and myocardin protein levels. The values from balloon injury have been normalized to values in sham group. ** $P < 0.01$ vs. sham. * $P < 0.01$ vs. miR-145. ‡ $P < 0.01$ vs. DM 14D. (n=6 per group).



Supplementary Figure S10. Wild type miR-145 and valsartan reduces intima area and increases carotid artery lumen size after carotid injury in diabetic rats. Upper panel, cross-sectional area of intima measured 14 d after carotid artery balloon injury. **P<0.01 vs. sham. *P<0.01 vs. 14 d. (n=4 per group). Lower panel, lumen size of carotid artery at 14 d after balloon injury. **P<0.01 vs. sham. *P<0.01 vs. 14 d. (n=4 per group).



Supplementary Figure S11. Masson's staining was used to assess the extent of vascular neointimal lesion formation in balloon-injured rat carotid arteries. Representative microscopic image of Masson's staining on extent of vascular neointimal lesion formation.