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Running title: EGCG augmented miR-145 through Klf4 in neointima

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Abstract

MicroRNA 145 (miR-145) is a critical modulator of vascular smooth muscle cell (VSMC) phenotyping and proliferation. Flavonoids have been studied extensively due to their diverse pharmacological properties, including anti-inflammatory effects. The aims of this study is designed to evaluate the atheroprotective effects on angiotensin II (Ang II)-induced miR-145 and Klf4/myocardin expression *in vitro* and *in vivo* of flavonoids, including (-)-epigallocatechin gallate (EGCG), chrysin, wogonin, silibinin, and ferulic acid. Ang II significantly reduced the miR-145 compared with the control VSMC groups; all the tested flavonoids increased miR-145 in the 100 nM concentration. Among the test compounds, EGCG showed the strongest augmenting effect on miR-145 and myocardin, however, it also abolished Ang II-induced Klf4. A [^{3}H]-thymidine incorporation proliferation assay demonstrated that EGCG inhibited Ang II-induced VSMC proliferation, and Klf4 siRNA presented with the similar results. Immunohistochemical analysis and confocal microscopy demonstrated increased Klf4 expression and the arterial lumen was narrowed after balloon injury 14 days. With the addition of EGCG (50 mg/kg) and Klf4 siRNA, neointimal formation was reduced by 40.7% and 50.5% compared with balloon injury 14 days; Klf4 expression also was attenuated. This study demonstrated EGCG increased miR-145 and attenuated Klf4, and ameliorated neointimal formation *in vitro* and *in vivo*. The

novel suppressive effect was mediated through the miR-145 and Klf4/myocardin pathways.

Key words: flavonoid, EGCG, angiotensin II, Klf4, microRNA 145, neointimal formation

Abbreviations: Angiotensin II, Ang II; microRNA 145, miR-145; Kruppel -like factor 4, Klf4; PCR, polymerase chain reaction; (-)-epigallocatechin-gallate, EGCG

1. Introduction

MicroRNAs (miRNAs) are short, noncoding RNAs containing 18–24 nucleotides. Dysregulation of miRNAs contributes to many diseases including cardiovascular diseases, and cancers [1,2]. miRNAs have emerged as an integral role in the transcriptional regulation of vascular smooth muscle cells (VSMCs) development, phenotyping, and function during vascular pathology [3]. Several studies have indicated that microRNA 145 (miR-145) is the most abundant miRNA in differentiated VSMCs and plays a crucial molecular role in VSMC phenotype switching [4]. Moreover, miR-145 was identified as a VSMC phenotypic marker, modulator of vascular neointimal formation control, direct stimulator of myocardin translation, and suppressor of the transcriptional repressors Kruppel-like factor 4 (KLF4) and 5 [5]. Overexpression of miR-145 resulted in reduced KLF4 and elevated myocardin expression in apolipoprotein E knockout (ApoE(-/-)) mice. Thus, miR-145 can be considered as a therapeutic target to limit atherosclerotic plaque morphology [6], and promotes differentiation and suppresses proliferation of VSMCs [7].

Angiotensin II (Ang II) contributes to the pathogenesis of atherosclerosis, and also affects on blood pressure, smooth muscle cell (SMC) growth, and antifibrinolytic activity [8]. Ang II, which is produced within vascular tissues, activates Ang II type 1 receptor (AT1R) leading to the accumulation of inflammatory cells, fibrosis, and the

proliferation or migration of VSMCs [9]. Moreover, Boettger et al. reported that the effects of miR-143/145 on smooth muscle differentiation and revealed angiotensin converting enzyme was crucial in special aortic target [10]. miR-143/145 KO mice exhibited the systolic blood pressure reduction in response to Ang II stimulation [11]. Investigation into the absence of the miR-143 and miR-145 showed a significant elevation in blood pressure and vascular tone in VSMC cell cultures, and also have a tendency to develop neointimal formation [12,13].

Flavonoids exist in vegetables, fruits, and herbal medicines. Flavonoids are a large family of polyphenolic compounds with a benzo-γ-pyrone structure and are present ubiquitous in plants [14]. Studies have recommended consuming botanical flavonoids to prevent degenerative diseases, including cardiovascular diseases and other age-related diseases [15]. Therefore, investigating how flavonoids protect against cardiovascular disease and understanding their regulatory mechanisms is important. We have reported that the effects of some flavonoids, namely chrysin, wogonin, ferulic acid, silibinin and EGCG (Fig. 1) on cardiovascular diseases, including angiogenesis, myocardial fibrosis, and neointimal formation [16-18]. Nevertheless, anti-neointimal potential of flavonoids on the regulation of miR-145 still remains unclear. The first aim of this investigation is to test whether chrysin, ferulic acid, EGCG, wogonin, or silibinin ameliorate the balloon injury induced

neointimal formations; and the second aim is to investigate the effects of flavonoids on molecular regulation of the miR-145/Klf4/myocardin pathway. The results of this study suggest that the flavonoids can protect against human cardiovascular diseases.

2.1. Materials and Methods

2.1. Chemical reagents

High purity EGCG (E4143, Sigma-Aldrich Chemical Company, St. Louis, MO, USA), chrysin, wogonin, ferulic acid (F3500, Sigma-Aldrich Chemical Company, St. Louis, MO, USA), and silibinin were purchased from Wako Pure Chemical Industry (Osaka, Japan). Dulbecco's modified Eagle's medium was purchased from GIBCO (Cat No. 31600-034, Life Technologies, Carlsbad, CA, USA). Ang II was obtained from Bachem (Cat No. H-1705, Bubendorf, Switzerland). Dimethyl sulfoxide (DMSO) were obtained from the Sigma-Aldrich Chemical Company (D2650, St. Louis, MO, USA). Penicillin, and streptomycin were obtained from the GIBCO (Cat No. 15140122, Life Technologies, Carlsbad, CA, USA). Finally, Matrigel basement membrane matrices were acquired from BD Biosciences (Cat No. 354234, Bedford, MA, USA).

2.2. Vascular smooth muscle cell (VSMC) cultures

Human coronary artery smooth muscle cells (HCASMCs) were produced by PromoCell GmbH (Order No. c-12511, Heidelberg, Germany). The cells were cultured in a smooth muscle cell growth medium (PromoCell GmbH (Order No. c-22062, Heidelberg, Germany) supplemented with 10% fetal bovine serum, 100

U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO2. Cells were grown to 80%-90% confluence in 100-mm culture dishes and were subcultured at a ratio of 1:2 Ang II antibody at 5 μg/mL (sc-7419, Santa Cruz Biotechnology Inc., California, USA), or flavonoids were added 30 min before Ang II stimulation to block the effects of humoral factors**.**

2.3. Quantification of microRNA 145

The micro RNA from cultured cells was isolated using mirVana™ miRNA Isolation Kit (Cat No. AM1560, Life Technologies, Carlsbad, CA, USA). The total RNA from arterial tissue was isolated using TRIzol Reagent (Cat No. 15596026, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. TaqMan MicroRNA Assays (Part No. 4440888, Applied Biosystems Inc., CA, USA) were used to quantitate miR-145 in all of our studies according to the manufacturer's instruction as described previously [19]. The expression levels of target miR145 were normalized according to U6 (Part No. 4395470, Applied Biosystems Inc., CA, USA).

2.4. Construction and delivery of miR-145 expression vector

The method of the 88 bp miR-145 precursor was constructed and delivered using the similar method as previous study [19]. In short, genomic DNA was amplified with forward primer AGAGAACTCCAGCTG and reverse primer GGCAACTGTGGGGTG. The 199 bp amplified product was digested with EcoRI

and BamHI restriction enzymes and ligated into pmR-ZsGreen1 plasmid vector (coexpression of miR-145 and green fluorescent protein; Cat. No. 632541 Clontech Laboratories, CA. USA) digested with the same enzymes. Antagomir-145 and mutant-miR-145 precursor construct were generated in a pmR-Zs Green1 plasmid vector. The mutated miR-145 was constructed by replacing a GTCCAGTT sequence with a CTGGACTT sequence. The constructed plasmid was transfected into cells using a low-pressure-accelerated gene gun (USA Patent No.7638322B2, Bioware Technologies, Taipei, Taiwan) following the protocol from the manufacturer. In brief, 2 μg of plasmid DNA was suspended in 20 μL of PBS and was delivered to the cultured cells at a helium pressure of 15 psi.

2.5. Fluorescence in situ hybridization assay for miR-145 detection in the VSMCs

Microscopic images indicated the presence of miR-145 (green color) in the VSMCs, but it was not detected in the control groups and scrambled probe. Fluorescence in situ hybridization assay was performed as in the previous study [16]. The cultured VSMCs were subjected to a brief acetylation reaction [66 mmol/L HCl, 0.66% acetic anhydride (v/v) and 1.5% triethanolamine (v/v) in RNase-free water]. Then, VSMCs were prehybridized at the hybridization temperature for 30 minutes in prehybridization solution which comprised of 50% deionized formamide, 5× sodium chloride/sodium citrate buffer, 1× Denhardt's solution, 500 μg/mL of yeast tRNA, and

0.01% Tween. The prehybridization solution was replaced with 200 μL of hybridization solution containing 10 pmol of the FAM-labeled LNA miR-145 probe (Product No. 88068-04, sequence 5′-AGGGATTCCTGGGAAAACTGGAC-3′, Exiqon, Vedbaek, Denmark). VSMCs were incubated for 90 min at the hybridization temperature and washed twice for 10 min in sodium chloride/sodium citrate buffer.

2.6. Western blot analysis

Western blotting was performed as previously described [19], using rat anti-Klf4 monoclonal antibody (Cat No. sc-20691, Santa Cruz Biotechnology, Inc., CA, USA) and rabbit anti-myocardin polyclonal antibodies (Cat No. sc-21561, Santa Cruz Biotechnology, Inc., CA, USA). The signals were visualized by chemiluminenescent detection. All western blot analyses were quantified using densitometry.

2.7. Real-time reverse transcription-polymerase chain reaction

Total RNA from the cultured fibroblasts was extracted using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. Real-time RT-PCR was performed as described previously [18]. The rat Klf4 primers were 5′-ATGCAGGCTGGGCAAAAC-3′ (forward) and 5′-CAGCCGTCCCAGTCACAGT-3′ (reverse); myocardin, 5′-CAGCTGGCTAACCAAGGCTT-3′ (forward) and 5′-TCAGAGAGTCTTCAGTCTTGGCACTA-3′; GAPDH,

5′-CATCACCATCTTCCAGGAGC-3′ (forward) and 5′-GGATGATGTTCTGGGCTGCC-3′ (reverse).

2.8. RNA interference

The cultured VSMCs were transfected with 800 ng of Klf4 siRNA oligonucleotides (Cat No. NM-004235, Sigma-Aldrich Chemical Company, MO, USA). Klf4 siRNA is a target-specific 20-25 nt siRNA designed to knockdown Klf4 gene expression, Klf4 siRNA, and scrambled siRNA sequences CUGUGGUAGUGGGCCCUA[dT][dT] (forward) and UAGGGCGCCACUACCACAG [dT][dT] (reverse), respectively.

2.9. Thymidine incorporation assay

The proliferation of VSMCs was determined using the [^{3}H]-thymidine incorporation assay. The thymidine incorporation assay is incorporated radioactive 3H-thymidine into new strands of chromosomal DNA during mitotic cell division. The method was performed as previously described [18]. In brief, VSMCs were seeded on a ViewPlate (Cat No.6005181, Perkin Elmer, Boston, MA, USA) for 60 minutes at a density of $5x10^3$ cells per well in serum-free medium and incubated overnight. Ang II, EGCG, Klf4 siRNA, and DMSO were then added to the plates. Thymidine uptake was measured by the addition of 500 nCi/mL [^{3}H]-thymidine (Cat No. NET027Z250UC, Perkin Elmer, Boston, MA, USA). The assay was performed in triplicate and each sample was normalized to the cells incubated without stimulation.

2.10. Migration assay

The migration assay was performed as previously described [18], with 250 μL of Matrigel was coated onto each well of cell culture (REF353097, BD Falcon, NJ, USA) insert with polycarbonate filters (pore size, 8 μm) and then incubated at 37°C for 30 min. Then, 5×10^4 VSMCs were implanted onto each of cell culture insert ‘s wells, and the EGCG-, Klf4 siRNA-, and miR145-treated medium was filled in the well to a total volume of 1 mL. After 18 h of incubation at 37°C and 5% CO2 in air, the Matrigel was removed. Then, cells that had migrated to the reverse side of the filter were stained with Liu's stain solution (Cat No. BA-4001, BA-4002, BaSO, New Taipei City, Taiwan). Using a photograph of four randomized visual fields, the percentage of cell migration was measured by counting the number of endothelial cells that had migrated through the membrane. The data are presented as percentages of the control group.

2.11. Balloon injury of the rat carotid artery

Animal experiments were approved and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 86-23, revised 2011), and also were approved by the *Affidavit of Approval of Animal Use Protocol by the Institutional Animal Care and Use Committee (IACUC)* of Shin Kong Wu Ho-Su Memorial Hospital. Adult Wistar

rats were anaesthetized with isoflurane (3%) and subjected to balloon catheter injury of the right carotid artery as described previously [18]. Briefly, a 2F Forgarty balloon catheter (Edwards Lifesciences Co., CA, USA) was inserted through the right external carotid artery, inflated and passed three times along the length of the isolated segment (1.5–2 cm in length); then, the catheter was removed. Klf4 siRNA was injected to the segment, and electric pulses were administered using CUY21 EDIT Square Wave Electroporator (Nepa Gene, Chiba, JAPAN) were administered with five pulses and five opposite polarity pulses at 250 V/cm, 50 ms duration, and 75 ms interval using Parallel fixed platinum electrode (CUY610P2–1, 1 mm tip, 2 mm gap). The injected siRNA was incubated for 10 min. After incubation, unbound siRNA was aspirated. The carotid artery was then tied off, and the neck was closed. The rats were sacrificed at 14 days after balloon injury, and the carotids were perfusion fixed at constant physiological pressure with 4% paraformaldehyde. The vessels were dissected, and embedded in paraffin blocks, and two cross-sections were cut at positions 1 and 2 cm upstream of the carotid bifurcation. The carotid artery was harvested and fixed in 10% formaldehyde and sliced into 5-μm-thick paraffin sections. Then an immunohistochemical study was performed as previously described [19]. Intimal, medial and adventitial cross-sectional areas were measured with image software (Image-Pro Plus, Media Cybernetics Inc. MD, USA). To test the effects of EGCG and

Klf4 siRNA on vessel remodeling after balloon injury, EGCG at 50 mg/kg of body weight was administered by oral gavage for 2 weeks following balloon injury.

2.12. Statistical analysis

The data were expressed as the mean ± SD of three replicates from four separate experiments. Statistical significance was determined by analysis of variance (GraphPad Software Inc., San Diego, CA, USA). Dunnett's test was used to compare multiple groups to a single control group. The Tukey-Kramer comparison test was used for pairwise comparisons between multiple groups after the ANOVA. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Angiotensin II attenuated miRNA 145 level in cultured VSMCs

To explore the effect of angiotensin II (Ang II) on miR-145, the miR-145 expression was detected in the cultured VSMCs in time-dependent manners. As shown in Fig. 2A, the miR-145 level was significantly attenuated in time-dependent manners after Ang II was induced in VSMC cultures.

3.2. Validity of miR-145, Klf4, and myocardin presentation in Ang II-induced VSMC cultures and Klf4 presentation in rat arteries

To validate the miR-145, Klf4, and myocardin presentation, the fluorescence immunohistochemical assay, Western blot, and real time PCR assay were performed. In Ang II-induced VSMC cultures and arterial transectional tissue, miR-145 was detected as a green dot (Fig. 2B). As shown in the miR-145 level was decreased in plateau 1 h (Fig. 2A), the downstream regulated protein and RNA analysis, Klf4 and myocardin were measured after Ang II (10 nM) induction in time-dependent manners (Fig. 2C and 2D). The Klf4 protein expression showed the peaked level in 6 h, and Klf4 mRNA presented earlier in 4 h. The Klf4 transferred to the rat carotid artery was monitored by using a high resolution CCD dissecting fluorescence microscope after balloon injury (Supplement 1). MTT assay revealed an absence of cytotoxicity in flavonoids and EGCG (data not shown).

3.3. Effects of EGCG and various flavonoids on miR-145 and Klf4 presentation in Ang II-induced VSMC cultures

To compare the inhibitory effects of EGCG on miR-145 and Klf4 with various flavonoids, the miR-145 level and Klf4 protein were detected. Figure 3A and 3B showed that EGCG (10^{-4} M) had a more significant inhibitory effect than other flavonoids did. With the same (10^{-4} M) concentrations of flavonoids (included chrysin, ferulic acid, silibinin, wogonin, and EGCG), EGCG presented the most inhibition of Klf4 and increase of the miR-145 level.

3.4. EGCG-induced miR-145 augmentation through Klf4 and myocardin regulation

To demonstrate the efficiency of EGCG on Klf4 and myocardin, in Western bot analysis, EGCG revealed the inhibition on Ang II-induced Klf4 protein expression in a concentration dependent manner (Fig. 4A). Treatment with EGCG, miR-145, and Klf4 siRNA revealed attenuated Klf4 protein expression in a similar manner to that in Ang II-induced VSMC cultures (Fig. 4B). Myocardin protein expression increased with treated with EGCG, miR-145 or Klf4 siRNA treatment after Ang II induction, the result also indicated that Klf4 abolished the downstream myocardin protein expression (Fig. 4C).

3.5. Attenuated proliferation of Klf4 siRNA, EGCG and miR-145 on VSMCs

We performed a [^{3}H]-thymidine incorporation assay to investigate the effects of Klf4 siRNA, miR-145, and EGCG on the Ang II-induced proliferation of VSMCs. Figure 5A showed that Ang II (10 nM) treatment significantly increased the VSMCs proliferation. Pretreatment with Klf4 siRNA, EGCG (10^{-4} M), or dominant miR-145 attenuated Ang II-induced VSMC proliferation (Fig. 5A).

3.6. Inhibitory effects of Klf4 siRNA, EGCG, and miR-145 on VSMC migration

We performed a cell migration assay to evaluate the effects of Klf4 siRNA, EGCG, and miR-145 on Ang II-induced phenotypic alterations of VSMCs. Ang II (10 nM) treatment significantly augmented the migratory activity of the VSMCs. However, pretreatment with Klf4 siRNA, EGCG (10^{-4} M), or dominant miR-145 diminished the migratory activity of Ang II-induced VSMCs (Fig. 5B).

3.7. EGCG and Klf4 siRNA attenuated neointima and increased lumen size after carotid artery balloon injury

Carotid artery balloon injury sustained for 14 days significantly increased the neointima of the intimal area and narrowed the vascular lumen, compared with the uninjured control group (Fig. 6A, left panel). Klf4 siRNA and EGCG treatment significantly attenuated the neointima of the intimal area after balloon injury. The neointima of the intimal area decreased by 50.5 % after Klf4 siRNA delivery, and 40.7 % after administrating EGCG treatment compared with balloon injury after 14

days (Fig. 6B, upper panel). EGCG and the Klf4 siRNA revealed significantly abolished Klf4 expression in the intimal area. Immunohistochemical staining demonstrated strong that Klf4-positive expression 14 days after carotid artery injury, whereas the intimal layer in the sham group exhibited less Klf4 expression (Fig. 6A, right panel). Klf4 siRNA and EGCG diminished the Klf4 and CD68 labeling signal intensity after carotid artery injury (Fig. 6B, lower panel).

4. Discussion

Flavonoids exist widely in vegetables and herbal medicines, and belong to the of large polyphenol family. Consuming botanical flavonoids has contributed to diminishing in cardiovascular diseases in the recent studies [20,21]. Increasing evidence from investigations suggested that flavonoids have the anti-atherosclerotic effects through the modulation of various pathways *in vitro* and *in vivo* models. Salvianolic acid A promoted p21 expression in umbilical artery smooth muscle cells (SMCs) through the cAMP/PKA/CREB signaling cascade *in vitro* and prevents injury-induced neointimal hyperplasia [22]. Furthermore, and nobiletin inhibited PDGF-BB-induced VSMC proliferation through attenuating reactive oxygen species (ROS) production and decreased the intima area in balloon-injured rat carotid arteries [23]. Balloon-injured carotid arteries and Ang II–induced VSMC were used to mimic the pathophysiologic conditions in the percutaneous angioplasty of vascular atherosclerosis. This study is the first to demonstrate the novel role of flavonoids and EGCG on molecular regulation of miR-145/Klf4/myocardin in Ang-II-induced VSMC cultures and in neointimal formation. Neointimal formation is a key element in atherosclerotic plaque and in arterial restenosis following balloon injury. Meanwhile, few studies have investigated the effects of flavonoids on balloon injury *in vitro* and *in vivo*.

Flavonoids are polyphenolic compounds with a benzo-??-pyrone structure, and have anti-inflammatory, anti-bacterial, and anti-cancer properties. Their functional activity was believed to the relation molecular hydrophobicity and charges on C atom at position 3 [24,25], Shen et al. have indicated that special flavonoid dihydrodaidzein (phytoestrogen isoflavone compound) halved the intimal response significantly, while tetrahydrodaidzein and dehydroequol showed no inhibitory effects [26]. Chen et al. have reported that flavonoid butein inhibited PDGF-induced VSMC proliferation and migration, and attenuated neointimal formation after percutaneous transluminal coronary angioplasty [27]. Treatment with special flavonoid resveratrol or in combination with resveratrol and quercetin, have exhibited the synergistic inhibition of VSMC proliferation and decreased the intima:media ratio [28]. Similarly, Guan et al. suggested the flavonoid apigenin suppressed PDGF-induced VSMC activation and neointima hyperplasia after vascular injury [29]. Taken together, these results on the from anti-neointimal formation of the various flavonoids showed that the benzo-??-pyrone structure played an important role in the improvement of cardiovascular diseases. Continuing our previous investigation, and these results revealed that EGCG ameliorated neointimal formation. However, in novelty, the results of the current study also showed flavonoids and EGCG diminished Klf4 expression through augmentation with miR-145 and myocardin in Ang II- induced

VSMC cultures.

A central role was played by miR-145 in SMC differentiation and it was upregulated by transcription factors such as myocardin and myocardin-related transcription factors [30]. Regarding *in vivo* therapeutic target to ApoE(-/-) knockout mice, Lovren et al. reported that VSMC-specific overexpression of miR-145 limited atherosclerotic plaque morphology and cellular composition, and that lentiviral delivery of mR-145 reduced KLF4 and elevated myocardin expression in aortas [6]. The Klf4 affected in SMC proliferation, meanwhile, myocardin augmented SMC differentiation [7]. Our *in vitro* Ang-II-induced VSMC culture data revealed in response to Klf4 and downstream myocardin regulation. Therefore, targeting miR-145 and myocardin to prevent VSMC differentiation and atherosclerosis may improve clinical outcomes for cardiovascular diseases. Other than in SMCs, or in cardiovascular diseases, miR-145 also plays an important role in other diseases. The effect of quercetin on the expression of miR-145 induced the apoptosis through activation of the extrinsic death receptor mediated intrinsic mitochondrial apoptotic pathways in SKOV-3 and A2780 human ovarian cancer cell lines [31]. Zaman et al. indicated that the proapoptotic gene TNFSF10 presentation was upregulated by miR-145 overexpression in prostate cancer PC3 cells, and that modulation of miR-145 may be an important therapeutic approach [32]. Our present data revealed that

flavonoids and EGCG augmented miR-145 and myocardin expression in VSMC cultures, and that EGCG also ameliorated neointimal formation and Klf4 presentation. These results indicated flavonoids and EGCG have miR-145 regulation properties.

According to habitual flavonoid intake studies, higher intake fruit flavonoids lead to lower risk of nonfatal myocardial injury and stroke in men [33]. Increasing evidence has indicated that the benefits of flavonoids in the prevention of cardiovascular diseases through various mechanism including anti-inflammation and regulation of novel miRNAs. Flavonoid puerarin facilitated the development of T-tubules through the suppression of miR-22 in murine embryonic stem cell-derived cardiomyocytes [34]. Arango et al. demonstrated that flavonoid apigenin exerted effective anti-inflammatory activity by reducing miR-155 expression in LPS-induced mice lungs and in macrophage cultures [35]. Flavonoid luteolin pretreatment conveyed anti-apoptotic effects after myocardial ischemia/reperfusion injury by decreasing miR-208b-3p and increasing Ets1 expression levels in rats [36]. Kim et al. documented that Kaempferol inhibited VSMC migration by modulating BMP-mediated miR-21 expression, and downregulating Dedicator of cytokinesis (DOCK) 4, 5, and 7 [37]. We have demonstrated that EGCG inhibited neointima and myocardial fibrosis. Therapeutic innovation targeting miR-145 may warrant further research. Following previous investigations, in this study, we further clarified that

flavonoids diminished neointimal formation through the miR-145/Klf4/myocardin pathway after carotid artery balloon injury.

In conclusion, these data demonstrated that flavonoids and EGCG attenuated vascular smooth muscle cell (VSMC) migration and proliferation through the miR-145/Klf4/myocardin pathway, thereby suppressing neointimal formation. Our study adds evidence to the claim that flavonoids and EGCG have therapeutic potential for the prevention and attenuation of atherothrombosis in future clinical applications.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Figure legends

Figure 1: Structures of chrysin, wogonin, ferulic acid, silibinin, and EGCG. Chrysin, molecular weight (MW): 254.24; wogonin, MW: 284.27; ferulic acid, MW: 194.19; silibinin, MW: 482.44; EGCG, MW: 458.37.

Figure 2: Effects of Ang II on the microRNA-145, Klf4, and myocardin in VSMC cultures. Exogenous administration of angiotensin II (Ang II) reduced the microRNA-145 (miR-145) expression. A: The level of miR-145 in VSMCs following exogenous Ang II administration was for various periods of time. B: In situ hybridization assay detection of the presence of miR-145 in the VSMCs. Microscopic images showing the presence of miR-145 (green color) in the cytoplasm of VSMCs. The scrambled probe did not detect the presence of miR-145. Original magnification, ×400. C: Western blots for exogenous administration of Ang II showed an increase in Klf4 and decrease in myocardin protein expression for various periods of time. D: Exogenous Ang II administration increased the Klf4 mRNA and decreased myocardin mRNA expression in time-dependent manners. *, $P < 0.05$ vs. control (n=4/group).

Figure 3: Effects of flavonoids on miR-145 and Klf4 following by Ang II administration in VSMC cultures. A: Effects of flavonoids (10^{-4} M), chrysin, ferulic

acid (FA), silibinin, wogonin, and various concentration of EGCG on the miR-145 expression in VSMCs following exogenous Ang II administration. EGCG augmented the miR-145 expression in a concentration-dependent manner. B: Representative Western blots of flavonoids showed a decrease in the Klf4 protein expression following by Ang II administration. EGCG attenuated the Klf4 protein following by exogenous Ang II administration in a concentration-dependent manner. *, $P < 0.05$ vs. Ang II-induced group (n=4/group).

Figure 4: Molecular regulation of EGCG on Klf4 and myocardin after Ang II administration in cultured VSMCs. Klf4 and myocardin expression in VSMCs subjected to Ang II administration for 6 h or control cells without Ang II administration, EGCG, and Klf4 siRNA. A: EGCG (10^{-4} M) and wild type miR-145 decreased the Klf4 protein expression following Ang II administration. B: EGCG, Klf4 siRNA and miR-145 augmented myocardin expression. *, $P < 0.05$ vs. Ang II-induced group (n=4/group).

Figure 5: Effects of EGCG, miR-145, and Klf4 siRNA on the proliferation assay in Ang II administrated VSMCs. A: [3H]-thymidine incorporation assay showing the proliferation of VSMCs following Ang II stimulation both in the presence and the

absence of either EGCG (10^{-4} M), dominant miR-145 and Klf4 siRNA. B: EGCG, miR-145, and Klf4 siRNA attenuated the migration activity. The assay was performed in triplicate, and the values from the induced VSMCs were normalized to those from the control cells. *, $P < 0.05$ vs. Ang II-induced group (n=4/group).

Figure 6: Arterial lumen immunohistochemnical staining of Klf4 in treatment with EGCG and Klf4 siRNA after balloon injury. Significantly increased immunoreactive signals were observed for Klf4 following balloon injury after 14 days. A: Left panel showed a cross-section of the aorta stained with the hematoxylin after EGCG (50 mg/kg) and Klf4 siRNA treatment. Right 2 panels showed double staining for Klf4 siRNA and CD68 (green color) related to smooth muscle cells actin labeling (red color). B: Upper panel showed quantitative analysis of the cross-sectional area of the Klf4 protein expression. Middle panel showed quantitative analysis of the cross-sectional lumen size measured; EGCG and Klf4 siRNA significantly increased the lumen size. Lower panel showed quantitative analysis of CD68 numbers measured 14 days after carotid balloon injury. *, $P < 0.05$ vs. the balloon injury induced group (n=4/group).

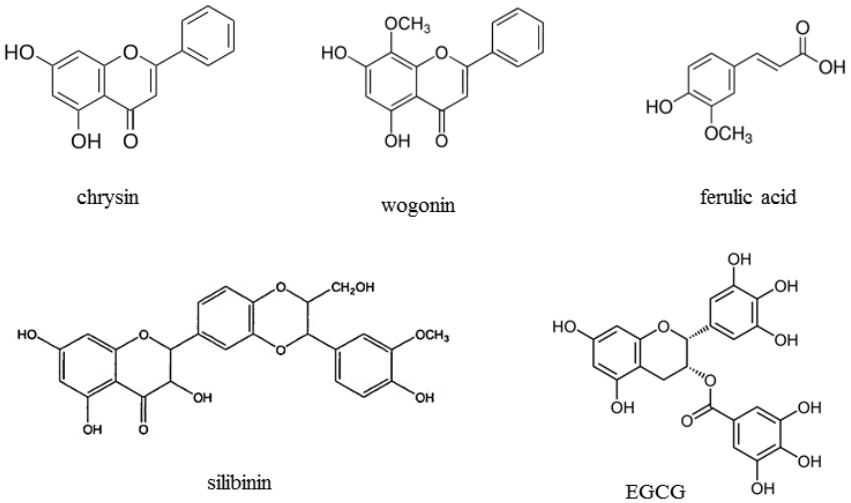


Figure 1

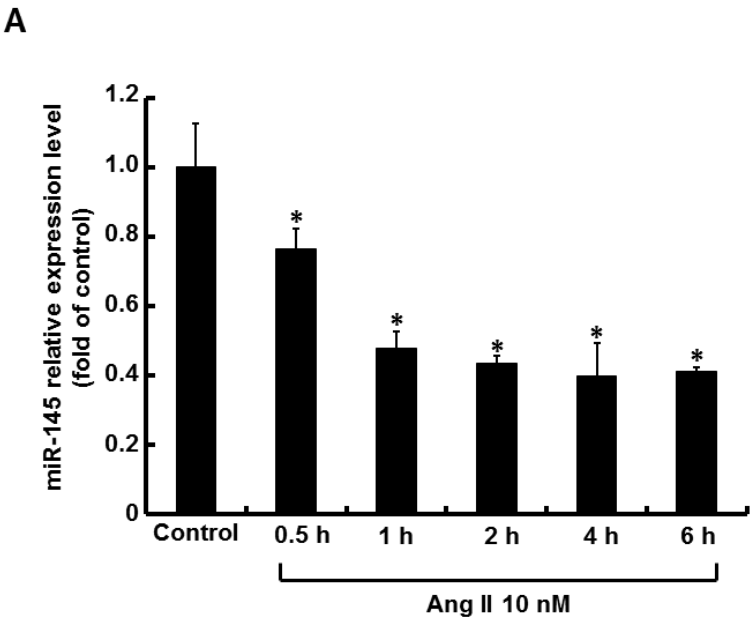


Figure 2a

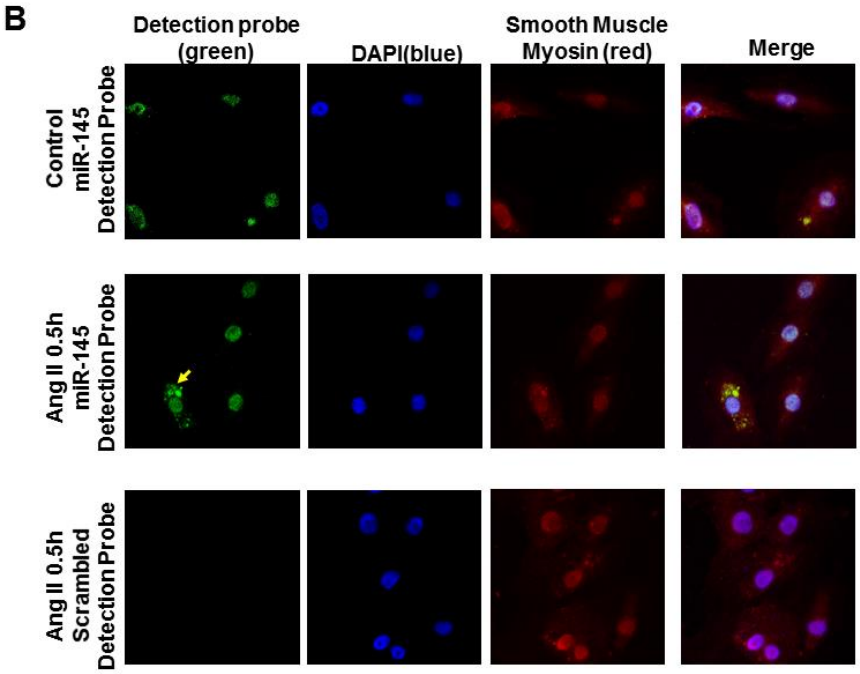


Figure 2b

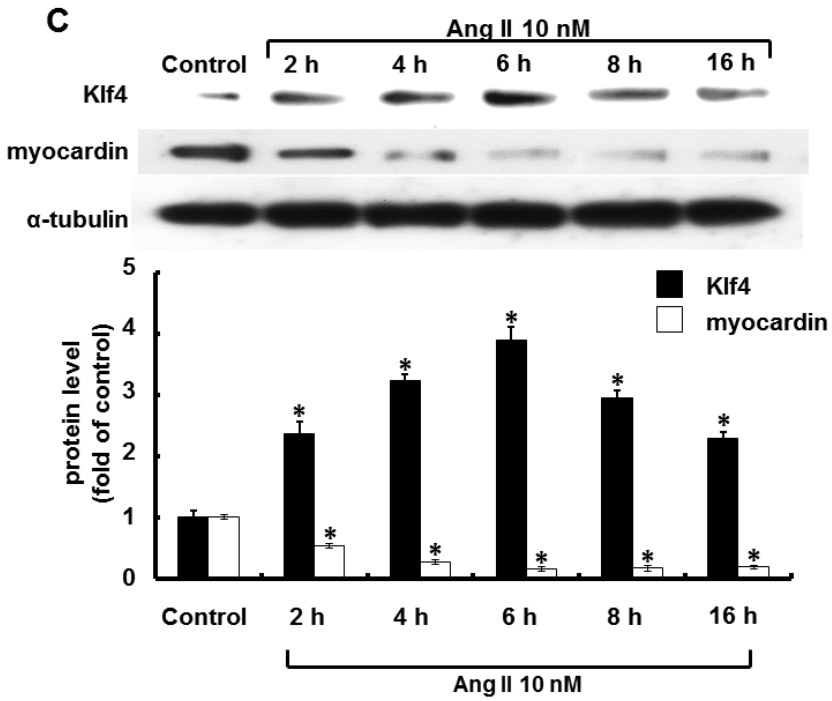


Figure 2c

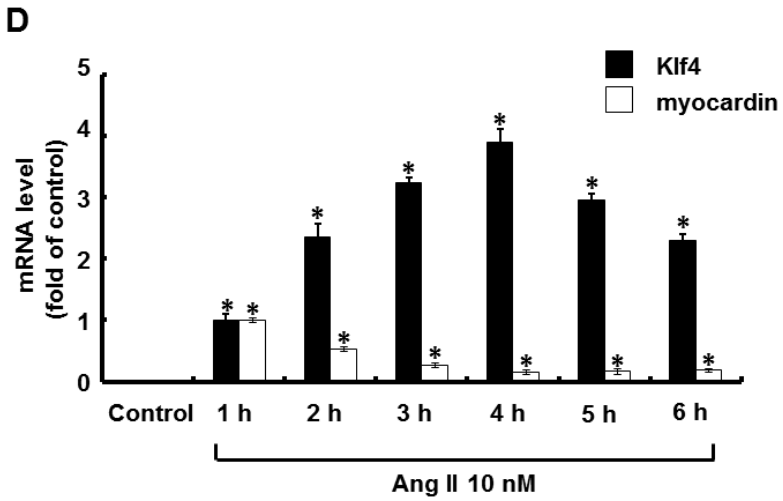


Figure 2d

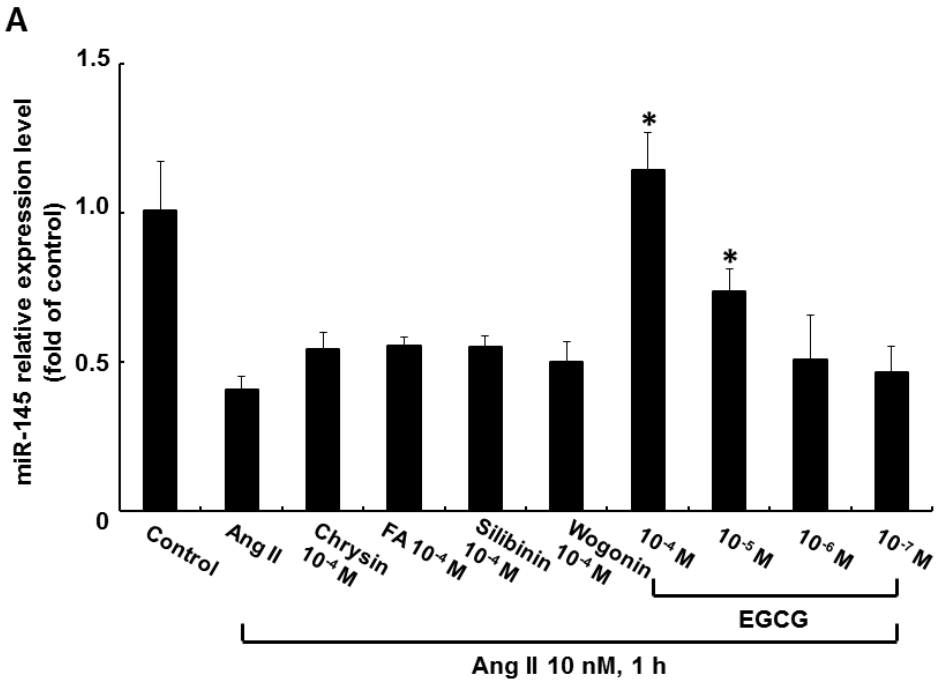
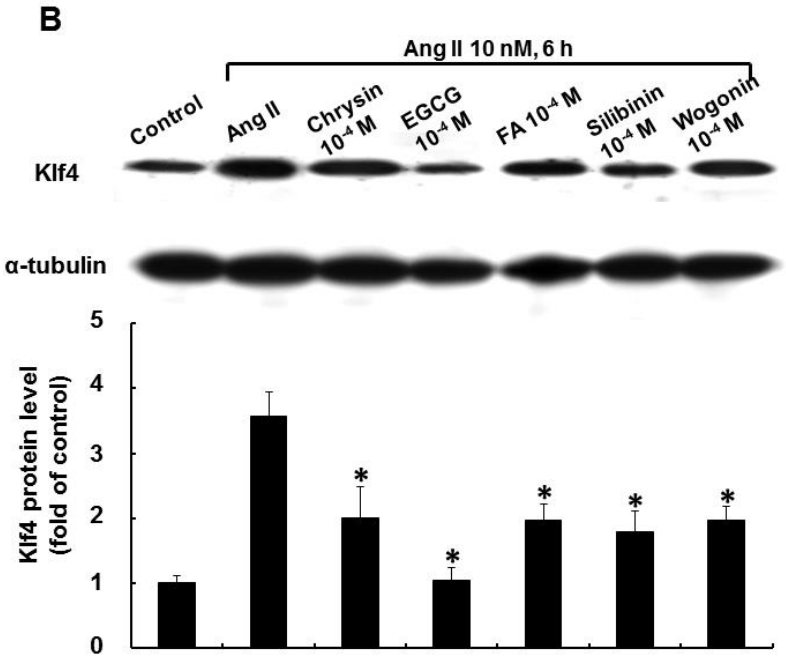


Figure 3a



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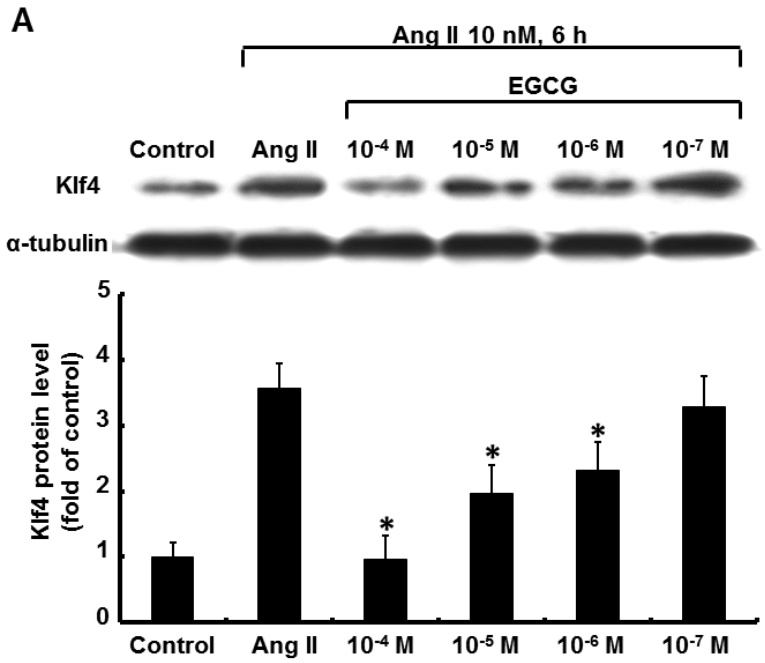
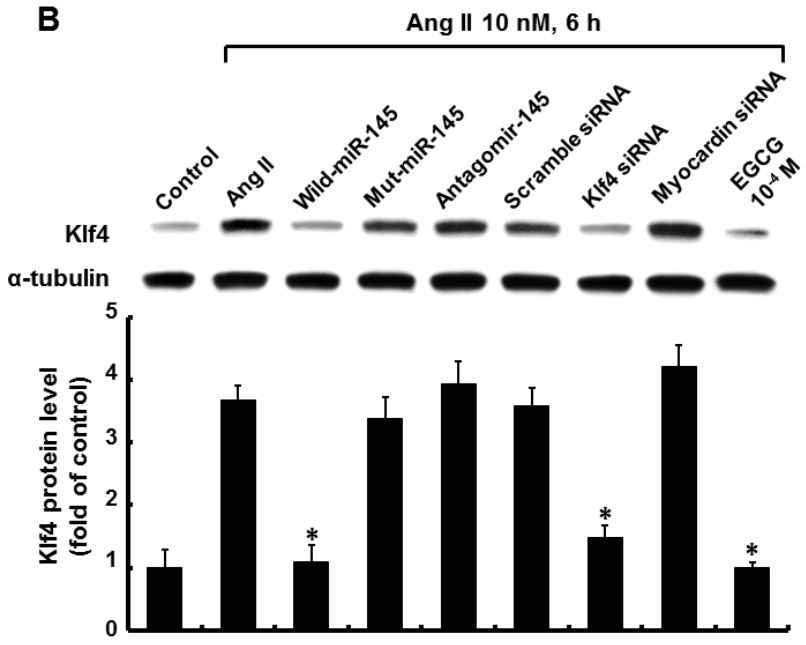


Figure 4a



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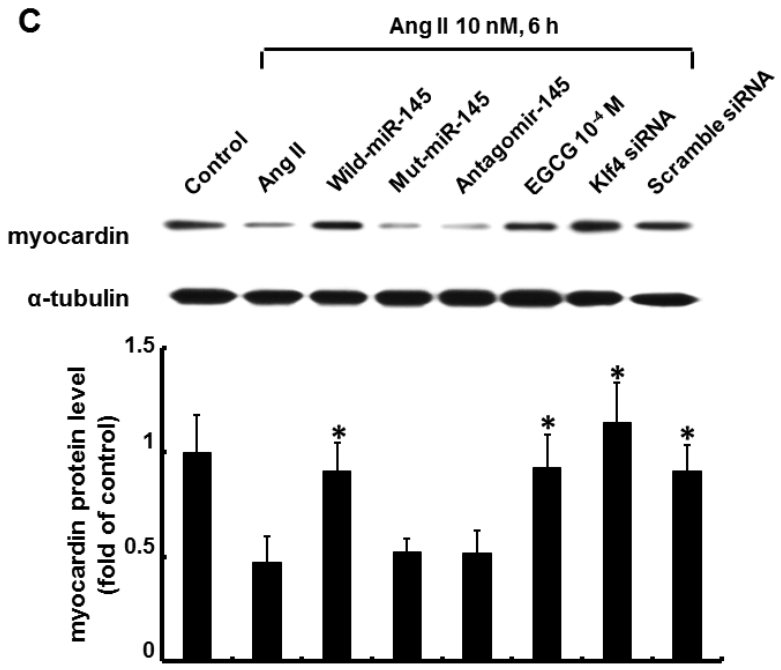

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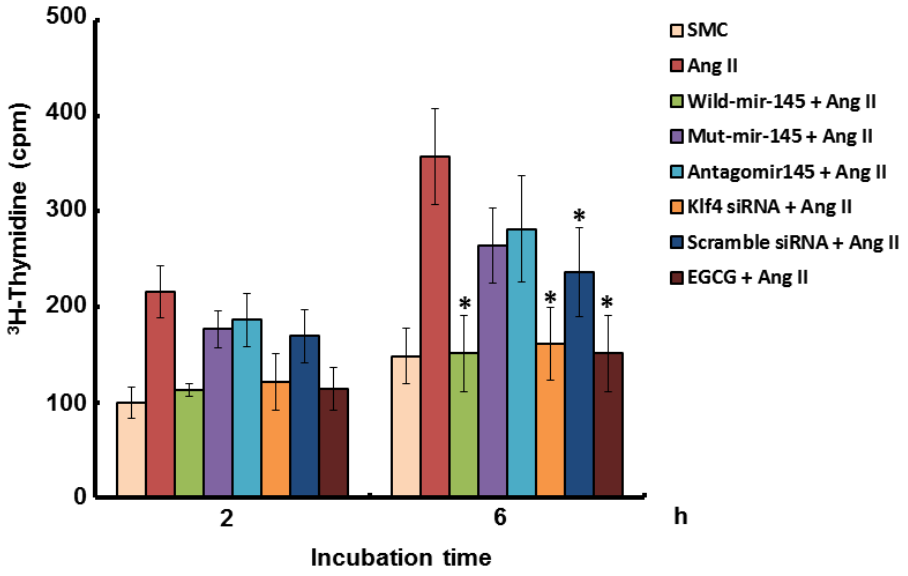

Figure 5a

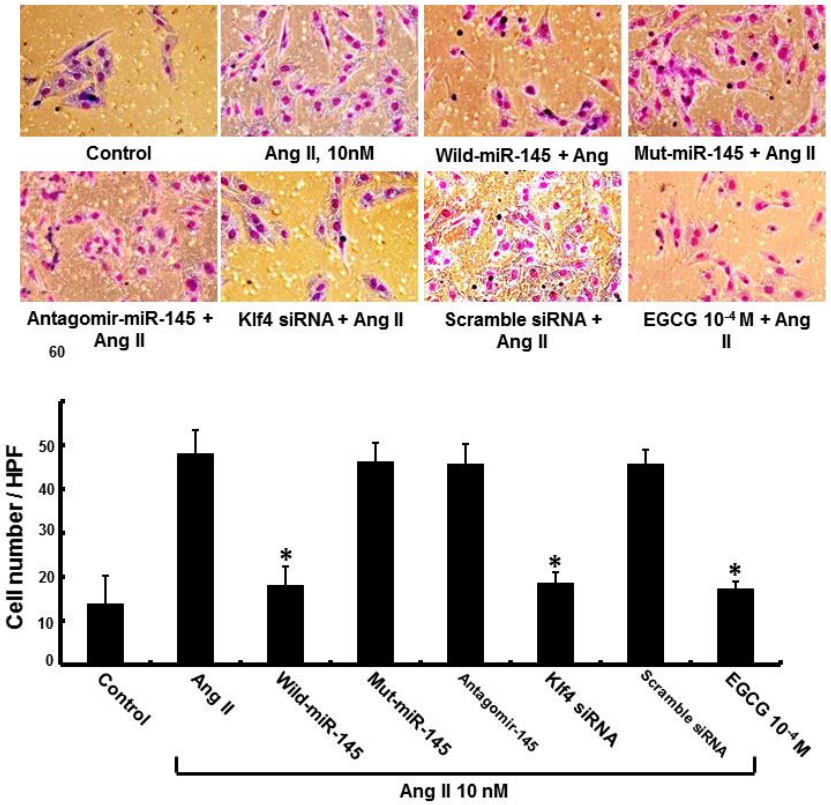

Figure 5b

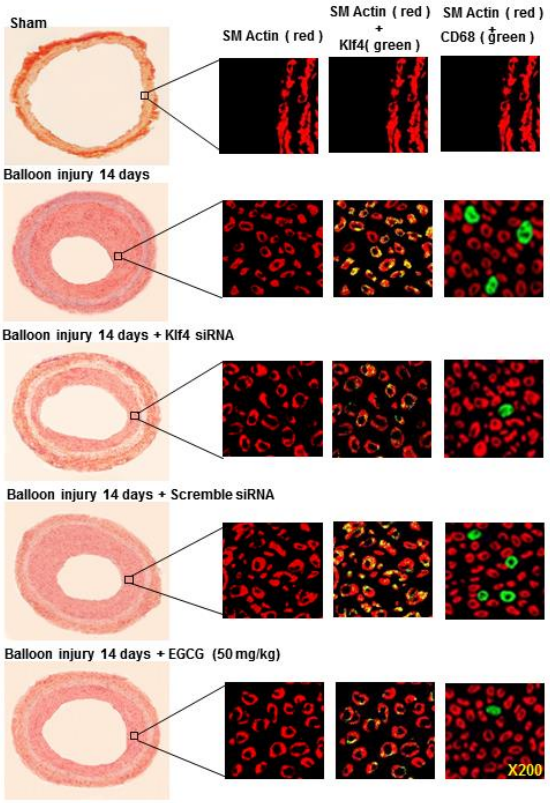

Figure 6a

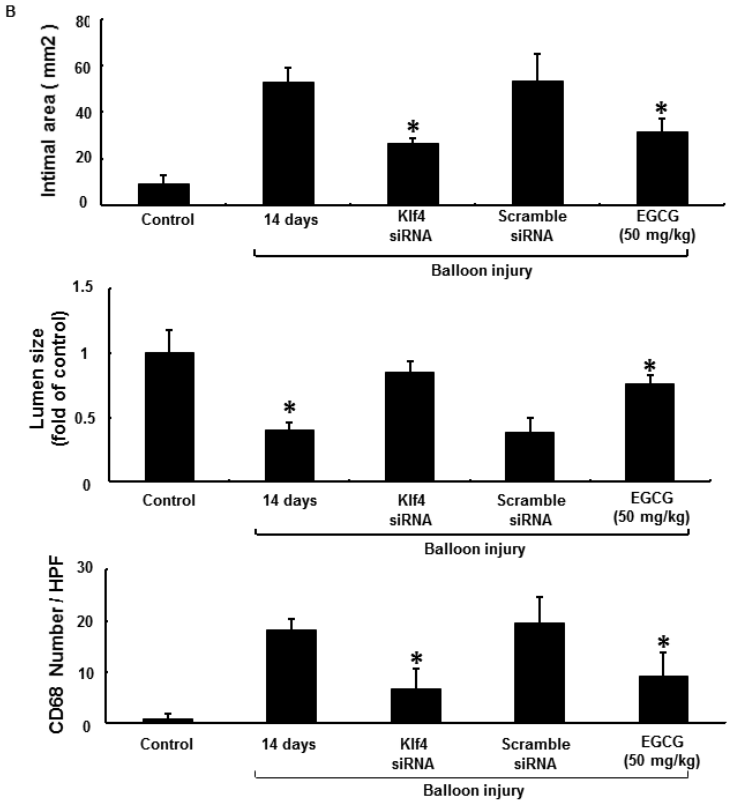

Figure 6b

Highlights:

- We have demonstrated that EGCG inhibited neointima and myocardial fibrosis. Therapeutic innovation targeting miR-145 may warrant further research. Following previous investigations, in this study, we further clarified that flavonoids diminished neointimal formation through the miR-145/Klf4/myocardin pathway after carotid artery balloon injury.
- In conclusion, these data demonstrated that flavonoids and EGCG attenuated vascular smooth muscle cell (VSMC) migration and proliferation through the miR-145/Klf4/myocardin pathway, thereby suppressing neointimal formation. Our study adds evidence to the claim that flavonoids and EGCG have therapeutic potential for the prevention and attenuation of atherothrombosis in future clinical applications.