Small RNA Sequencing Reveals MicroRNAs That Modulate Angiotensin II Effects in Vascular Smooth Muscle Cells

Received for publication, November 9, 2011, and in revised form, February 27, 2012. Published, JBC Papers in Press, March 19, 2012, DOI 10.1074/jbc.M111.322669

Wen Jin1*, Marpadga A. Reddy2*, Zhuo Chen5, Sumanth Putta5, Linda Lanting5, Mitsuo Kato5, Jung Tak Park3, Manasa Chandra1, Charles Wang4, Rajendra K. Tangirala1, and Rama Natarajan3†§

From the †Irell and Manella Graduate School of Biological Sciences, ‡Division of Molecular Diabetes Research, Department of Diabetes, and §Functional Genomics Core of the Department of Molecular Medicine, Beckman Research Institute of City of Hope, Duarte, California 91010 and the 1Division of Endocrinology, Diabetes, and Hypertension, David Geffen School of Medicine, University of California, Los Angeles, California 90095

Background: Role of microRNAs in angiotensin II-mediated vascular smooth muscle cell dysfunction is unclear. Results: Angiotensin II up-regulates miR-132 in VSMC. miR-132 induces MCP-1 partly via targeting PTEN, activates CREB, and regulates genes related to cell-cycle and motility. Conclusion: miR-132/212 is a novel modulator of Ang II actions. Significance: microRNAs may serve as new drug targets for Ang II-mediated cardiovascular diseases.

Angiotensin II (Ang II)-mediated vascular smooth muscle cell dysfunction plays a critical role in cardiovascular diseases. However, the role of microRNAs (miRNAs) in this process is unclear. We used small RNA deep sequencing to profile Ang II-regulated miRNAs in rat vascular smooth muscle cells (VSMC) and evaluated their role in VSMC dysfunction. Sequencing results revealed several Ang II-responsive miRNAs, and bioinformatics analysis showed that their predicted targets can modulate biological processes relevant to cardiovascular diseases. Further studies with the most highly induced miR-132 and miR-212 cluster (miR-132/212) showed time- and dose-dependent up-regulation of miR-132/212 by Ang II through the Ang II Type 1 receptor. We identified phosphatase and tensin homolog (PTEN) as a novel target of miR-132 and demonstrated that miR-132 induces monocyte chemoattractant protein-1 at least in part via PTEN repression in rat VSMC. Moreover, miR-132 overexpression enhanced cyclic AMP-response element-binding protein (CREB) phosphorylation via RASA1 (p120 Ras GTPase-activating protein 1) down-regulation, whereas miR-132 inhibition attenuated Ang II-induced CREB activation. Furthermore, miR-132 up-regulation by Ang II required CREB activation, demonstrating a positive feedback loop. Notably, aortas from Ang II-infused mice displayed similar up-regulation of miR-132/212 and monocyte chemoattractant protein-1, supporting in vivo relevance. In addition, microarray analysis and reverse transcriptase-quantitative PCR validation revealed additional novel miR-132 targets among Ang II-down-regulated genes implicated in cell cycle, motility, and cardiovascular functions. These results suggest that miR132/212 can serve as a novel cellular node to fine-tune and amplify Ang II actions in VSMC.

Angiotensin II (Ang II)2 exerts various pathophysiological effects in the vessel wall, leading to not only vasoconstriction and hemodynamic effects but also proinflammatory, growth-promoting, and vascular remodeling events (1–3). Ang II activates vascular smooth muscle cells (VSMC) in the vessel wall and induces VSMC proliferation and hypertrophy (4, 5), key functions associated with hypertension, restenosis, and atherosclerosis (6). Moreover, Ang II induces the expression of cytokines such as interleukin-6 (IL-6) (7, 8), monocyte chemotactrant protein-1 (MCP-1) (9) in VSMC and osteopontin (10) in the arterial wall, key players involved in monocyte recruitment, neointimal formation, and atherosclerosis (2, 11).

The role of Ang II-mediated VSMC dysfunction in the pathogenesis of hypertension and atherosclerosis has been extensively studied in cultured VSMC and in vivo models. These diverse effects of Ang II are mostly mediated by the Ang II type 1 receptor (AT1R), leading to the activation of several key signaling pathways, including mitogen-activated protein kinases (MAPKs) (3) and transcription factors NF-κB (7) and cAMP response element-binding protein (CREB) (6). Ang II activates CREB through phosphorylation at the Ser-133 residue in a MAPK-dependent manner, and this is functionally associated with VSMC hypertrophy and inflammation (7, 8). However, the molecular mechanisms involved in Ang II-mediated VSMC dysfunction remain incompletely understood.

Growing evidence suggests that microRNAs (miRNAs) play critical roles in cardiovascular development and disorders (12).

* This work was supported, in whole or in part, by National Institutes of Health Grants NIH R01 HL087864 and NIH RO1 HL106089 (NLHB; to R. N.).

†§ This article contains supplemental Fig. S1 and Tables S1–S3.

All genomic data reported in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) database, smRNA seq GEO submission (GSE35664), and Affymetrix data GEO submission (GSE35627).

1 To whom correspondence should be addressed: Dept. of Diabetes, Beckman Research Institute of City of Hope, 1500 East Duarte Rd., Duarte, CA 91010. Tel.: 626-256-4673 (ext. 62289); Fax: 626-301-8136; E-mail: RNatarajan@coh.org.

2 The abbreviations used are: Ang II, angiotensin II; MCP-1, monocyte chemoattractant protein-1; CREB, cAMP responsive element-binding protein; pCREB, phosphorylated CREB; RASA1, p120 Ras GTPase-activating protein 1 (p120RasGAP); PTEN, phosphatase and tensin homolog; VSMC, vascular smooth muscle cell(s); RVMSC, rat VSMC(s); miRNA, microRNA; AT1R, Ang II type 1 receptor; qPCR, quantitative PCR; IPA, ingenuity pathway analysis; smRNA-Seq, small RNA deep sequencing; 132-M, miR-132 mimic; NC-M, control mimic; 132-I, miR-132 inhibitor; Ctrl, control.
miRNAs are non-coding RNA molecules 20–22 nucleotides in length that can negatively regulate gene expression and affect diverse biological processes. Typically, the seed sequences of mammalian miRNAs base pair with binding sites in the 3′-untranslated region (3′-UTR) of target mRNAs, leading to translational inhibition and/or mRNA degradation of these target genes (13). miRNA biogenesis and maturation begins with transcription of pri-miRNA by RNA polymerase II or III followed by pri-miRNA nuclear cleavage to generate pre-miRNA, which is transported to the cytoplasm and further processed by the RNase Dicer to form mature miRNA. Then these miRNAs are incorporated into the RNA-induced silencing complex and interact with target mRNAs to fine-tune gene regulation under diverse pathophysiological conditions (13). Recent studies with VSMC have identified functional roles for various miRNAs such as miR-143 and miR-145 which regulate VSMC differentiation (14), contractility, and Ang II-induced hypertension (15), miR-21 (16) and miR-31 (17) that regulate VSMC proliferation, and miR-125b that promotes proinflammatory responses in VSMC under diabetic conditions (18). However, the role of miRNAs in Ang II-mediated VSMC dysfunction has not yet been investigated.

In this study we utilized the recent technology of small RNA deep sequencing (smRNA-Seq) to profile Ang II-regulated miRNAs in rat VSMC (RVSMC) and also examined their functional relevance. We observed that Ang II increased the expression of miR-132 and miR-212 cluster (miR-132/212) in RVSMC in vitro and in aortas of Ang II-infused mice in vivo. We demonstrated that phosphatase and tensin homolog (PTEN) is a novel target of miR-132 and that miR-132/212 play key roles in Ang II-induced MCP-1 gene expression in RVSMC. Furthermore, we uncovered a positive feedback loop mechanism between Ang II-induced CREB activation and miR-132 expression in RVSMC. In addition, we observed that the predicted targets of miR-132 that were also down-regulated by Ang II have biological functions related to cell cycle, cell motility, and cardiovascular diseases.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All animal studies were performed according to Institutional Animal Care and Use Committee-approved protocols. RVSMC were isolated from thoracic aortas of male Sprague-Dawley rats (Charles River) by enzymatic digestion (8) and cultured in M199 medium supplemented with 10% FBS and antibiotics. FACS analysis showed 99.2% cells stained positive for smooth muscle cell specific marker α-actin (data not shown). RVSMC were serum-depleted for 48 h in M199 medium supplemented with 0.2% BSA before Ang II stimulation unless mentioned otherwise.

**smRNA-Seq**—Total RNA was used for the construction of small RNA libraries, cluster generation, and then deep sequencing (at the Beckman Research Institute Sequencing Core) using the Alternative v1.5 Protocol (Illumina Inc., San Diego, CA) with minor optimization. Briefly, 0.5 μg of total RNA was ligated to sRNA 3’ adapter (AUCUCGUAUUGCUCUCUCGCUU) using T4 RNA Ligase 2, truncated (New England Biolabs, Ipswich, MA) at 22 °C for 1 h, and subsequently ligated to the SRA 5’ adapter (GUUCAGAGUUCACAGUCCGACGAUC) with T4 RNA ligase (New England BioLabs, Ipswich, MA) at 20 °C for 1 h. The adaptor-linked RNA was converted to single-stranded cDNA using Superscript II reverse transcriptase (Invitrogen) and RT-Primer (5′-CAAGCAGAAGACGGCATACGA-3′), and then amplified with Phusion DNA Polymerase (Finzymes Thermo Scientific, Pittsburgh, PA) for 12 cycles using primers (5′-CAAGCAGAAGACGGCATACGA-3′; 5′-AAATGATACGCGACACCCGAGGTCCAGACG-15673

---

Ang II-induced miR-132 in VSMC

**Data Analysis of smRNA-Seq**—SmRNA-Seq data were analyzed as described earlier (18) with some modifications. Raw data in FASTQ format generated from the Illumina pipeline was aligned against Rat Nov. 2004 (rn4) assembly using Novoalign software. The 3’-adapter sequence of the raw reads was first trimmed by Novoalign, and reads with 16 or more bases were aligned to rat genome. For reads aligned to multiple locations on the genome, one aligned region was randomly selected for counting the number of reads (supplemental Table S1) as described (19). Genomic locus of each rat mature miRNA was generated by aligning rat mature miRNA sequences (miRBase v14) to rn4 assembly without allowing mismatches. Repetitive regions were not filtered out. For each sample, an aligned read was annotated to a specific genome feature in the order of mature miRNA, miRNA precursor, rRNA, tRNA, other non-coding RNA, RfSeq gene, and intergenic region if it completely fell in the specified region with a 10-bp extension on both ends. Specifically, aligned reads were first annotated to rat mature miRNA (v14, miRBase) genomic loci. The reads that could not be annotated to mature miRNA regions were processed further to annotated pre-miRNA genomic loci (v14). Remaining reads were then annotated to non-coding RNA sequences other than miRNAs including rRNA and tRNA using a rat non-coding RNA annotation file downloaded from UCSC genome browser. Finally, the reads that were not annotated to any non-coding RNA regions were assigned to Refseq genes, and the remaining reads were defined as intergenic regions. For each sample, the reads corresponding to the mature miRNA genomic loci (including 10-base extension on both ends) were counted to obtain expression levels of total miRNAs (supplemental Table S2). The resulting miRNA expression dataset was further normalized by scaling the total mature miRNA counts in each sample to 8.5 million. Differentially expressed miRNAs induced by Ang II at the indicated time points were selected using the following criteria: (a) reads in at least one sample were above 256 and (b) showed at least a 1.5-fold change between Ang II-treated samples versus untreated.

**Cluster Analysis of Differentially Expressed miRNAs**—In each sample the raw read counts for each differentially expressed miRNA was scaled by the total aligned miRNA reads and log2-transformed. The transformed data were then mean-centered and subjected to unsupervised hierarchical clustering analysis using Euclidean distance as dissimilarity metric and average linkage.
Ang II-induced miR-132 in VSMC

Analysis of Potential Targets of Differentially Expressed miRNAs—Potential targets of differentially expressed miRNAs were identified using online bioinformatics tools TargetScan Human 5.1 (20), miRanda (21), and Diana-microT V3.0 (22). For each miRNA, only the targets that were predicted by at least two tools and conserved across rat, mouse, and human were selected. We pooled 2067 genes representing potential targets of all the differentially regulated miRNAs. This pooled target gene set was subsequently analyzed by DAVID v6.7 (david. abcc.ncifcrf.gov) to obtain enriched biological processes and Ingenuity pathway analysis (IPA) for potential pathways relevant to cardiovascular functions.

RNA Isolation and Gene Expression—RNA was extracted using miRNAseasy columns (Qiagen, Inc., Valencia, CA). Gene expression analysis (miRNA and mRNA) was performed by reverse transcriptase-qPCR using the miScript Reverse Transcription kit and SYBR Green PCR kit (Qiagen) using primer sequences listed in supplemental Table S3. In this method, mature miRNAs present in RNA samples were first polyadenylated by poly(A) polymerase and then reverse-transcribed into cDNA using oligo-dT primer with a universal tag. Subsequently, miRNAs were amplified using specific mature mRNA sequences (designed by us or ordered from Qiagen) as forward primers and the universal primer provided in the kit as the reverse primer in the qPCR reaction. Expression levels of pri-miRNA of miR-132/212 were analyzed by Taqman pri-miRNA assay (Applied Biosystems, Carlsbad, CA).

Western Blotting—Preparation of protein lysates and Western blotting were performed as described earlier with some modifications (8, 18). Antibodies against Ser-133-phosphorylated CREB (pCREB), total CREB, and PTEN were purchased from Cell Signaling Technology (Beverly, MA). The RASA1 antibody was purchased from R&D systems (Minneapolis, MN).

VSMC Transfection—Transfection of VSMC was performed using Nucleofector II (Lonza, Basel, Switzerland), according to the manufacturer’s instructions. RVSMC were trypsinized and resuspended in Basic Nucleofector Solution for mammalian smooth muscle cells at 1 × 10^6 cells/ml. Next, 100 µl of cell suspension (1 × 10^6 cells) was mixed with miRNA mimic, 2′-O-methyl-modified hairpin inhibitor oligonucleotides, ON-target plus siRNA (Dharmacon products, Thermo Fisher Scientific Inc., Waltham, MA), or plasmids as indicated, and electroporated in a Lonza-certified cuvette with Nucleofector II device using the Nucleofector program D-33 optimized for RVSMC (optimization data not shown). In some experiments VSMC were transfected with plasmids expressing miR-132/212-resistant PTEN (plasmid 10750, Addgene, Cambridge, MA) (23) or RASA1 (24).

VSMC were also transfected using X-tremeGENE siRNA Transfection Reagent (Roche Applied Science) for luciferase assays according to the manufacturer’s protocol. Transfected cells were harvested for RNA, protein, and luciferase assays as described (18) at the indicated times.

Transduction of VSMC with Lentiviruses—The lentiviral particles were generated in the Vector Laboratory at the Beckman Research Institute. Briefly, HEK 293T cells at a density of 4 × 10^6/10-cm culture dish were transfected with pCMV-G (containing the VSV-G gene), pCgp (containing the HIV-1 gag and pol genes), and pCMV-rev-2 (containing the rev gene) and precursor miRNA expression plasmid or miRNA inhibitor expression plasmid (GeneCopia, Rockville, MD) using calcium phosphate co-precipitation. Virus-containing supernatant was harvested at 24 and 36 h after transfection, and the titer was determined in HT1080 cells by flow cytometry analysis of respective fluorescence markers (25). To transduce RVSMC with lentiviral vectors expressing miRNA precursors and inhibitors, lentiviral particles were added to cells in the medium containing Polybrene (5 µg/ml), and medium was changed 24 h later.

Construction of 3′-UTR Reporter Plasmids—3′-UTRs of potential miR-132/212 targets were amplified from rat genomic DNA using specific primers and cloned downstream of firefly luciferase gene into Apal and XbaI sites of pZeo/luc vector (26) and Xhol and NotI sites of psiCHECK-2 vector (Promega, Madison, WI). Cloned 3′-UTR fragments were verified by DNA sequencing. The PTEN 3′-UTR region was amplified using primers 5′-CTGTCGACGCTAGATGATTGAATGGT-CCTCAG-3′ and 5′-TGAACCGCGCCACGTTAGAG-TCAACTCTGCAAATAC-3′. RASA1 3′-UTR region was amplified using primers 5′-CATTCTAGACACACCTTTC-CACATTCCAGTGAGT-3′ and 5′-CACGGGCCCATA-CCTTCCCTTATAACCTTGGTG-3′.

Ang II Infusion—Male C57BL/6J mice (from The Jackson Laboratory, Bar Harbor, ME) were implanted with Alzet osmotic mini-pumps (Model 2004) filled with either PBS or Ang II (2.5 µg/min/kg) for 2 weeks. At the end of the experiment, aortas were collected, and adventitia was carefully removed before subsequent RNA extraction and gene expression analysis by RT-qPCR.

Affymetrix Gene Array Experiments and Data Analysis—Microarray hybridization and data acquisition were carried out by the Functional Genomics and Bioinformatics Cores at the Beckman Research Institute of City of Hope. Briefly, biotinylated cDNA derived from total RNA was hybridized with Affymetrix Rat Gene 1.0 ST arrays representing 27,342 well characterized genes. Three independent biological replicates were performed for each culture condition. Raw intensity data in CEL file format were imported to Partek Genomics Suite (Partek Inc., St. Louis, MO) and then preprocessed and normalized by Robust Multichip Average method. Differentially expressed genes at different time points in RVSMC stimulated with Ang II relative to control (untreated) were subsequently identified using analysis of variance method in Partek.

Statistical Analysis—PRISM software (Graphpad, San Diego, CA) was used for data analysis. All data shown are the means ± S.E. p < 0.05 was regarded as statistically significant based on Student’s t-tests (between two groups) or analysis of variance (between multiple groups).

RESULTS

Identification of Ang II-regulated miRNAs Using High Throughput smeRNA-Seq—Evidence shows that various miRNAs play key roles in regulating VSMC differentiation and proliferation, but the involvement of miRNAs in Ang II-mediated effects in VSMC is still largely unknown. To identify dif-
Ang II-induced miR-132 in VSMC

Differentially expressed miRNAs in response to Ang II in cultured RVSMC, we performed smRNA-Seq (22–30 nucleotide) with small RNA libraries generated using total RNA extracted from untreated (control) or Ang II-stimulated (100 nM) RVSMC at various time points. We identified several differentially expressed miRNAs using the stringent criteria that each miRNA has more than 256 reads in at least one sample, and the normalized reads show a more than 1.5-fold change after Ang II treatment compared with the control group (Fig. 1A). Ang II treatment triggered changes in some candidate miRNAs including up-regulation of miR-132, miR-212, miR-129, miR-21*, and miR-7a that were further validated by RT-qPCR (Fig. 1B). The levels of miR-145, known to be highly expressed in VSMC (14), were not significantly altered by Ang II (Fig. 1B).

Because each miRNA target prediction algorithm (TargetScan, miRanda, and Diana-microT V3.0) has its own advantages, targets theoretically predicted by multiple bioinformatics software and having conserved seed sequences are more likely to be true targets. We, therefore, used these bioinformatics tools and selected the targets that were predicted by at least two databases and conserved across rat, mouse, and human. These selected targets (2067 genes) were pooled and subjected to further bioinformatics analysis to identify biological processes and signaling networks regulated by them. Gene Ontology analysis by DAVID revealed significant enrichment of biological processes such as cell proliferation, cell migration, and cell adhesion, relevant to inflammatory and cardiovascular diseases (Fig. 1C). Furthermore, Ingenuity pathway analysis identified key signaling pathways including ERK/MAPK signaling and PTEN and CREB signaling, known to be involved in Ang II-mediated signaling networks regulated by them. Therefore, we further investigated the functional roles of these miRNA in VSMC and Ang II actions. Time course experiments showed that miR-132/212 levels started to increase as early as 3 h after Ang II stimulation and remained elevated up to 24 h after stimulation compared with control (Ctrl) RVSMC (Fig. 2A). Dose-response experiments showed that Ang II potently induced both the miRNAs at as low as 1 nM that remained elevated up to 10,000 nM (Fig. 2B). In all subsequent experiments, RVSMC were stimulated with Ang II at a concentration of 100 nM unless otherwise indicated. Furthermore, pretreatment with AT1R blocker Losartan (10 μM) markedly blocked Ang II-induced miR-132/212 in RVSMC (Fig. 2C), demonstrating the role of AT1R activation.

We also examined whether the increased expression of these miRNAs in response to Ang II could be due to increases in primary transcript levels. We found that Pri-miR-132/212 levels were markedly up-regulated (by >20-fold) in response to Ang II. The levels increased by 1 h after Ang II stimulation and remained elevated up to 24 h, suggesting the involvement of transcriptional regulation in RVSMC (supplemental Fig. S1).

Because miR-132 and miR-212 share the same seed sequence (AACAGUC), indicating they have the same targets, and mature miR-132 is more abundant than miR-212 in RVSMC, we examined the functions and targets of this cluster mostly through modulating miR-132 levels in subsequent studies.

miR-132 Targets PTEN—TargetScan algorithm predicted four miR-132/212 binding sites in the 3’-UTR of PTEN in rat (Fig. 3A), and three of them were highly conserved in human and mouse, suggesting PTEN could be a potential target of miR-132. Because PTEN can have several protective effects in VSMC (27, 28), we examined whether Ang II and miR-132 could down-regulate PTEN in RVSMC. Results showed that Ang II significantly inhibits the expression of the putative miR-132 target PTEN in RVSMC (Fig. 3B). Next, we observed that transfection of RVSMC with miR-132 mimic (132-M) oligonucleotides also significantly down-regulated both PTEN mRNA (Fig. 3C) and protein (Fig. 3D) levels relative to those transfected with control mimic (NC-M) oligonucleotides. To verify if PTEN is a direct target of miR-132, we cloned the PTEN 3’-UTR containing four miR-132 binding sites (nucleotides 1119–2972) downstream of firefly luciferase reporter and co-transfected it along with 132-M or NC-M oligonucleotides into RVSMC. Luciferase assays were performed 48 h post-transfection. Results showed significantly reduced luciferase activity in 132-M-transfected cells compared with NC-M (Fig. 3E). In contrast, co-transfection with miR-132 hairpin inhibitor oligonucleotides (132-I) that inhibit miR-132 increased PTEN 3’-UTR activity relative to control inhibitor NC-1 (Fig. 3F). These results clearly demonstrate that PTEN is a direct and novel target of miR-132 in RVSMC.

miR-132 Enhances MCP-1 Gene Expression in Part through PTEN Inhibition—Next, we tested the functional relevance of PTEN down-regulation. Previous studies in mouse VSMC showed that PTEN knockdown increased the expression of MCP-1 (29), which is also known to be induced by Ang II in VSMC. In addition, PTEN overexpression was reported to suppress Ang II-induced MCP-1 mRNA expression (30). Therefore, we next examined the effect of miR-132 overexpression on MCP-1 gene expression. Results showed that MCP-1 expression was significantly increased in RVSMC transfected with 132-M oligonucleotides relative to cells transfected with NC-M (Fig. 4A). Our data also showed increased levels of MCP-1 mRNA in RVSMC transfected with lentiviral vectors expressing precursor miR-132 (LV-132-M) relative to the control vector (LV-Scr-M) (Fig. 4B). In contrast, inhibition of endogenous miR-132 by lentiviral-expressed anti-miR-132 (LV-132-I) significantly blocked both basal and Ang II-induced MCP-1 mRNA levels relative to control vector (LV-Scr-I) (Fig. 4C), further confirming the role of miR-132 in MCP-1 expression. Moreover, PTEN gene silencing by specific siRNAs (siPTEN) (Fig. 4D) (mimicking miR-132 actions) indeed significantly increased MCP-1 expression relative to control non-targeting oligonucleotide (siNTC) in RVSMC (Fig. 4E). In contrast, the overexpression of PTEN without its 3’-UTR (Fig. 4F) significantly suppressed 132-M-induced MCP-1 mRNA expression (Fig. 4G), further supporting the role of PTEN in miR-132-
FIGURE 1. Identification of Ang II-regulated miRNAs in RVSMC using smRNA-Seq. A, unsupervised hierarchical clustering of differentially expressed miRNAs (>1.5-fold) in VSMC treated with Ang II (100 nM) at the indicated time points compared with Ctrl samples is shown. For each miRNA, red indicates high expression, and green indicates low expression relative to the average of all samples. RNA isolated from Ctrl and Ang II-treated samples was subjected to smRNA-Seq analysis as described under “Experimental Procedures.” B, RT-qPCR validation of some differentially expressed miRNAs in RVSMC treated with Ang II for 24 h is shown. Results were expressed as % of Ctrl (*, p < 0.05; **, p < 0.01; ***, p < 0.001, n = 6). C, a pie chart shows relative distribution of the Biological Processes highly enriched among predicted target genes of Ang II-regulated miRNAs identified by DAVID v6.7 (57) analysis (modified Fisher exact test, p < 0.05; actual values are shown). D, shown is IPA analysis of potential signaling pathways enriched among predicted targets of Ang II-regulated miRNAs.
induced regulation of MCP-1. Together, these data suggest that the down-regulation of PTEN by miR-132 could be one of the mechanisms involved in augmenting MCP-1 expression in Ang II-treated RVSMC.

miR-132 and CREB Form Positive Regulatory Feedback Loop—Activation of CREB through MAPK-mediated Ser-133 phosphorylation plays an important role in Ang II-induced VSMC dysfunction, including hypertrophy, extracellular matrix gene expression, IL-6 up-regulation, and vascular remodeling (8, 31–33). Evidence shows that miR-132 could increase CREB phosphorylation by targeting inhibitors of upstream Ras-Raf1-ERK signaling, including ras GTPase-activating protein 1 (RASA1 or p120RasGAP), in rat neuronal cells and HEK293 cells (34). RASA1 was also validated as a miR-132 target in endothelial cells, and this was associated with vascular endothelial growth factor-induced angiogenesis (24). Therefore, we tested if miR-132 can also regulate CREB activity in RVSMC. We first observed that transfection with 132-M significantly increased phospho-CREB (pCREB) levels without changes in the internal controls including total CREB (tCREB) and /-H9252-actin (Fig. 5A, left panel). Next, we examined the effect of miR-132 inhibition on pCREB levels. Results showed a significant reduction in Ang II-induced pCREB levels in RVSMC transfectected with a mixture of 132-I and 212-I relative to RVSMC transfected with NC-I (Fig. 5B, left panel). The ratio of pCREB to /-H9252-actin determined by a calibrated densitometer is shown in the right panels (Fig. 5, A and B). Fig. 5C shows the conserved miR-132 and miR-212 binding sites in the rat RASA1 3/-H11032-UTR. Interestingly, both RASA1 mRNA and luciferase activity of the reporters containing rat RASA1 3’-UTR (nucleotides 117–1308) were also significantly down-regulated by 132-M (Fig. 5, D and E, respectively), supporting RASA1 as a target of miR-132 also in RVSMC. To further verify the role of RASA1 in CREB phosphorylation, we next examined whether expression of
Ang II-induced miR-132 in VSMC

RASA1 without the miR-132/212 binding sites can attenuate Ang II-induced PTEN down-regulation partly through PTEN down-regulation. Therefore, the results suggested that Ang II infusion can significantly induce miR-132/212, enhance MCP-1, and attenuate PTEN gene expression, supporting the in vivo relevance.

Unbiased Microarray Screening Reveals That Subset of Genes Down-regulated by Ang II in VSMC Are Predicted Targets of miR-132—To determine if Ang II induced up-regulation of miR-132 can affect its target genes related to VSMC functions or cardiovascular disease, we next adopted a more unbiased profiling approach. Because mammalian miRNAs can function through repression of mRNA levels (38), we performed Affymetrix transcriptome profiling of RNA obtained from VSMC treated with Ang II for various time periods at which miR-132 was also up-regulated. Then we selected putative miR-132 target genes down-regulated in Ang II-treated VSMC (Fig. 7B) as likely to be directly repressed by miR-132 in response to Ang II. This approach revealed that 49 potential miR-132 target genes were down-regulated in Ang II-treated VSMC (Fig. 7B). RASA1 is not represented in this heatmap because it was decreased by 17% in the original microarray, whereas for this comparison our selection criteria was at least 20% downregulation by Ang II. IPA was next used to uncover potential biological processes mediated by these 49 down-regulated genes. Results showed that these miR-132 targets could be associated with processes such as regulation of cell cycle, cell morphology, and cellular movement as well as cardiovascular system development, function, and disease (Fig. 7C). We next tested five genes enriched in these biological functions for further validation by RT-qPCR in VSMC. To examine this, we knocked down CREB protein levels by transfection with siRNAs targeting CREB (siCREB) relative to non-targeting control oligonucleotides (siNTC) (Fig. 5G, tCREB). Levels of pCREB were also reduced (Fig. 5G, pCREB). Furthermore, CREB down-regulation by siCREB led to significant inhibition of both basal and Ang II-induced expression of mature miR-132 and miR-212 (Fig. 5H). Some minimal residual Ang II effects may due to either the incomplete knockdown of CREB or that other transcription factors are also involved in up-regulating miR-132 expression (36). These results demonstrate that CREB activation is required for miR-132/212 up-regulation by Ang II and also the operation of a positive feedback loop that can amplify miR-132 expression and CREB activation in RVSMC.

Ang II Infusion Increases miR-132/212 in Vivo in Mice Aorta—We next examined the in vivo relevance using aortas from Ang II-infused mice. Alzet miniosmotic pumps were implanted subcutaneously in male C57BL/6J mice to deliver Ang II or the vehicle PBS for a 2-week period according to reported protocols (37). At the end of the infusion period, aortas were collected from both PBS- and Ang II-infused mice, adventitia were removed, and gene expression was examined by RT-qPCR. Results showed that the expression of miR-132 (Fig. 6A) and miR-212 (Fig. 6B) as well as the downstream inflammatory marker MCP-1 (Fig. 6C) were significantly increased in aortas from Ang II-infused mice compared with PBS treated mice. Furthermore, expression of the miR-132 target PTEN showed a decreased trend that was, however, not statistically significant (Fig. 6D). Overall, these results suggest that Ang II infusion can significantly induce miR-132/212, enhance MCP-1, and attenuate PTEN gene expression, supporting the in vivo relevance.
Both RVSMC treated with Ang II and those transfected with miR-132-M. Results demonstrated that all these five potential miR-132 targets were significantly down-regulated by Ang II at the indicated time points relative to control cells (Fig. 7D). Furthermore, transfection of miR-132 oligonucleotides also attenuated their expression in RVSMC compared with those transfected with control NC-M (Fig. 7E). Together these data suggest that, by targeting multiple genes with key relevant functions, miR-132 may have multiple roles in Ang II-mediated VSMC dysfunction.

**DISCUSSION**

In this study we used the smRNA-Seq method for the first time to profile Ang II-regulated miRNAs in VSMC and also observed that some of these miRNAs can act as novel mediators and modulators of Ang II actions in VSMC. Importantly, we demonstrated the up-regulation of miR-132/212 cluster by Ang II both in vitro in RVSMC and in vivo in Ang II-infused mice. We also showed that miR-132 could modulate Ang II-mediated MCP-1 gene expression in VSMC at least in part through its novel target PTEN. Moreover, our results demonstrated the role of AT1R and CREB in miR-132/212 regulation and established a positive feedback loop between CREB activation and miR-132/212 expression in RVSMC, potentially through down-regulation of the miR-132 target RASA1 (Fig. 8). Furthermore, we used a more unbiased approach to demonstrate that other potential targets of miR-132 could modulate processes associated with cardiovascular disease functions such as cell cycle and cell motility.

miR-132 and miR-212 in RVSMC transfected with the indicated oligonucleotides under basal conditions or after stimulation with Ang II (100 nM) for 6 h. Results were expressed as % of siNTC basal (**, p < 0.01; ***, p < 0.001, n = 6).
Ang II-induced miR-132 in VSMC

Recent advances in next generation sequencing technologies have led to high throughput and robust approaches such as smRNA-Seq that can quantify miRNA expression genomewide and also lead to the discovery of new miRNAs. These approaches have provided highly useful and novel information with human and mouse cells. However, similar analysis in rat cells has been difficult because the rat genome annotation is still relatively incomplete. Our studies provide the first smRNA-Seq data base catalogue of RVSMC. We observed that Ang II can activate CREB and down-regulate RASA1, key regulatory molecules associated with aberrant angiogenesis and endothelial dysfunction by fine-tuning target gene expression (12).

Bioinformatics approaches indicated that the predicted targets of differentially expressed miRNAs could be enriched in several well known Ang II-regulated biological processes and signaling pathways. DAVID analysis suggested that these predicted targets of Ang II-regulated miRNAs are likely to be involved in cell proliferation, migration, and translation regulation processes (Fig. 1C) relevant to VSMC hyperplasia and hypertrophy associated with hypertension, restenosis, and atherosclerosis. Several signaling pathways activated by Ang II contribute to VSMC proliferation, hypertrophy, and migration, including tyrosine kinases (6), MAPKs, Akt, and CREB (6, 32). IPA analysis of Ang II-responsive miRNA targets revealed enrichment of several of these pathways including ERK/MAPK, PI3K/Akt, PTEN, and CREB signaling (6, 32). This was further supported by experimental evidence that miR-132 could activate CREB and down-regulate RASA1 and PTEN, key signaling molecules associated with aberrant VSMC gene expression and signaling. Further experimental studies are needed to confirm the functional roles of other Ang II-regulated miRNAs and their predicted targets.

In this study we focused on the conserved miR-132/212 cluster as they were highly induced by Ang II in vitro and in vivo. Moreover, although the functions of these miRNAs have been demonstrated in different biological contexts such as neuronal system, endocrine system, innate immunity, and vascular endothelium, their role in VSMC is not known. These miRNAs are transcribed from an intergenic region on rat chromosome 10 and have been shown to be regulated by upstream CREB binding sites in neuronal cells (35). Our study demonstrated that CREB knockdown decreased Ang II-induced miR-132/212 expression, suggesting a direct role for CREB in mature miR-132/212 cluster regulation by Ang II. Furthermore, miR-132 in turn could activate CREB, demonstrating a positive feedback loop.
loop between CREB and miR-132/212 in RVSMC (Figs. 5 and 8). A similar regulatory mechanism was also recently implicated in cocaine-stimulated neurons (34). Ang II-induced CREB activation plays a key role in inflammatory and extracellular matrix gene expression and hypertrophy (8, 31–33). Thus, miR-132/212 could play an important role in these processes by amplifying CREB signaling. Evidence also shows a key role for CREB in VSMC phenotypic modulation (40); however, the role of miR-132/212-induced CREB activation in these events awaits further investigation.

Reports show that miR-132 has diverse effects on inflammatory responses in different cell types. These include inhibition of inflammatory gene expression by targeting proinflammatory acetylcholinesterase in neurons (41) and coactivator histone acetyl transferase p300 in monocytes (42). In contrast, miR-132 enhanced inflammatory gene expression through targeting Sirtuin 1, a histone deacetylase that inhibits NF-κB activation in preadipocytes and adipocytes (43). We observed that miR-132 mimic increased MCP-1 expression in VSMC, whereas its inhibitor blocked Ang II-induced MCP-1 expression. However, miR-132 did not have a significant effect on another proinflammatory cytokine IL-6, suggesting some level of specificity. MCP-1 is a major inflammatory mediator and chemoattractant (11) that also promotes VSMC migration and proliferation (44, 45). Ang II-induced MCP-1 promotes the accumulation of macrophages and lymphocytes in the atherosclerotic lesions of animal models (46, 47). We also identified PTEN as a direct target of miR-132. Furthermore, PTEN gene silencing by specific siRNAs increased MCP-1 expression, whereas its overexpression inhibited both Ang II- and miR-132-induced MCP-1, suggesting PTEN down-regulation as one of the mechanisms involved in miR-132-induced MCP-1 expression. However, increases in MCP-1 expression by PTEN gene silencing was much lower than that induced by miR-132 transfection, suggesting that other pathways such as NF-κB activation through targeting Sirtuin 1 (43) and RASA1 might also be involved. Previous studies showed that PTEN gene silencing or VSMC-specific PTEN knockdown increased inflammatory gene expression, neointima formation, growth, migration, and vascular remodeling (27, 29, 30, 48). Thus, miR-132 may augment Ang II-mediated VSMC dysfunction by modulating some of these processes. Hydrogen peroxide (H₂O₂), a downstream effector of Ang II actions (49), plays a key role in Ang II-induced MCP-1 expression in VSMC (9). Furthermore, H₂O₂ directly stimulates CREB activation (50) and increases miR-132 (51) in VSMC. Based on our data that CREB is required for Ang II-induced miR-132 transcription, it is possible that Ang II-generated H₂O₂ might also promote MCP-1 expression through increasing miR-132 in RVSMC.

In vivo relevance was supported by our observations that Ang II infusion could significantly induce miR-132/212 and enhance MCP-1 expression in mice aortas. Under the same conditions, the miR-132/212 target PTEN was also attenuated, although not statistically significant. This may be due to the fact that, besides smooth muscle cells, aortas also contain other cell types. Because miRNA effects are known to be cell type-specific, it is possible that PTEN was not efficiently targeted by miR-132/212 in cell types other than VSMC.

We also identified additional novel miR-132 targets by microarray profiling of Ang II down-regulated genes in RVSMC coupled with bioinformatics analysis. This method is advantageous because it can identify multiple putative VSMC-specific miR-132 targets among the down-regulated genes in an unbiased manner. However, we might have also missed some true miR-132 targets due to the stringent criteria used as well as other mechanisms such as translation inhibition of target mRNAs. Interestingly, IPA analysis of the miR-132 targets identified in this comparative analysis indicated their potential regulatory roles in cell growth, morphology, and movement (functions related to Ang II effects in VSMC and cardiovascular disease). Similar to PTEN, Foxo3 (52) was reported to inhibit VSMC cell cycle progression and, hence, neointimal hyperplasia. Moreover, Zeb2 (53), Arhgef11 (54), Sox4 (55), and Ssh2 (56) have been shown in other systems to modulate cell cycle, cellular apoptosis, or morphological changes, which are again Ang II-related biological processes. Therefore, biological functions mediated by these potential miR-132 targets are worthy of further investigations in the future.

In summary, we have demonstrated a new role for miRNAs in Ang II actions and in particular the up-regulation of miR-132 and miR-212 by Ang II in vitro and in vivo as well as the role of miR-132 in key Ang II responses including CREB activation and inflammatory gene expression. We also demonstrated a positive feedback loop between CREB signaling and miR-132, suggesting that this circuitry may fine-tune and amplify Ang II-mediated gene expression (Fig. 8). Because miRNAs generally regulate multiple genes, miR-132 and miR-212 up-regulation could have profound effects on several genes involved in VSMC dysfunction and thus serve as potential therapeutic targets for Ang II-mediated cardiovascular diseases.

Acknowledgments—We thank Dr. Ali Ehsani and Dr. Amy Leung for helpful discussions and Mei Wang for technical assistance. We are extremely grateful to Dr. Harry Gao, Charles Warden, Dr. Zheng Liu, and Dr. Yate-Ching Yuan (Beckman Research Institute Sequencing and Functional Genomics Core) for tremendous help with the miRNA-Seq and microarray work. We also appreciate the help from Dr. Fiting-Kuan Yee and the Vector Laboratory for the lentiviral vector preparation. We thank Merck & Co., Inc. (Whitehouse Station, NJ) for the gift of Losartan, Dr. David Cheresh and Dr. Anand Sudarshan (University of California, San Diego) for the gift of miRNA-resistant RASA1 plasmid, Dr. William Sellers’ help for providing the PTEN plasmid and its control plasmid through Addgene, Debra Rateri (University of Kentucky, Lexington) for advice on the Ang II infusions in mice, and Dr. Mary Weiser-Evans (University of Colorado, Denver, CO) for help with the PTEN vector.

REFERENCES
1. Daugherty, A., and Cassis, L. (2004) Angiotensin II-mediated development of vascular diseases. Trends Cardiovasc. Med. 14, 117–120
2. Marchesi, C., Paradis, P., and Schiffrin, E. L. (2008) Role of the renin-angiotensin system in vascular inflammation. Trends Pharmacol. Sci. 29, 367–374
3. Duff, J. L., Marrero, M. B., Paxton, W. G., Schieffer, B., Bernstein, K. E., and Berk, B. C. (1995) Angiotensin II signal transduction and the mitogen-activated protein kinase pathway. Cardiovasc. Res. 30, 511–517
4. Gibbons, G. H., Pratt, R. E., and Dzau, V. J. (1992) Vascular smooth muscle
cell hypertrophy vs. hyperplasia. Autocrine transforming growth factor-β1 expression determines growth response to angiotension II. J. Clin. Invest. 90, 456–461

5. Natarajan, R., Gonzales, N., Lanting, L., and Nadler, J. (1994) Role of the lipoygenase pathway in angiotension II-induced vascular smooth muscle cell hypertrophy. Hypertension 23, 1142–1147

6. Mehta, P. K., and Griendling, K. K. (2007) Angiotension II cell signaling. Physiological and pathological effects in the cardiovascular system. Am. J. Physiol. Cell Physiol 292, C82–C97

7. Han, Y., Runge, M. S., and Brasier, A. R. (1999) Angiotensin II induces interleukin-6 transcription in vascular smooth muscle cells through pleiotropic activation of nuclear factor-κB transcription factors. Circ. Res. 84, 695–703

8. Sahar, S., Reddy, M. A., Wong, C., Meng, L., Wang, M., and Natarajan, R. (2007) Cooperation of SRC-1 and p300 with NF-κB and CREB in angiotension II-induced IL-6 expression in vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 27, 1528–1534

9. Chen, X. L., Tummala, P. E., Olbrych, M. T., Alexander, R. W., and Medrano, D. J. (1998) Angiotensin II induces monocyte chemotaxatrant protein-1 gene expression in rat vascular smooth muscle cells. Circ. Res. 83, 952–959

10. Bruemmer, D., Collins, A. R., Noh, G., Wang, W., Territo, M., Liu, X., and Zhang, C. (2011) MiR-132 in VSMC cell hypertrophy and embedded tissue specimens. Small RNA deep sequencing of paired frozen and formalin-fixed, paraffin-embedded tissue specimens. MicroRNA profiling of clear cell renal cell carcinoma by whole-genome human microRNA biogenesis. J. Biol. Chem. 286, 42371–42380

11. Eiselt, S. C., and Nicolas, M. (2009) Tumor suppressor protein is linked to inhibition of the phosphatidylinositol-3-kinase/Akt pathway. Proc. Natl. Acad. Sci. U.S.A. 96, 1102–1105

12. Anand, S., Majeti, B. K., Acevedo, L. M., Murphy, E. A., Mukthavaram, R., Scheppke, L., Huang, M., Shields, D. J., Lindquist, J. N., Lapinski, P. E., King, P. D., Weiss, S. M., and Cherses, D. B. (2010) MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. Nat. Med. 16, 909–914

13. Bone, J. T., and Bartel, D. P. (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 19, 92–105

14. Norin, B. J., Enright, A. J., Aravin, A., Tuschl, T., Sander, C., and Marks, D. S. (2004) Human microRNA targets. PLoS Biol. 2, e363

15. Maragkakis, M., Reczko, M., Simossis, V. A., Alexiou, P., Papadopoulos, G. L., Dalamagas, T., Giannopoulou, G., Goumas, G., Kouris, E., Kourtis, K., Vergoulis, T., Koziris, N., Sellis, T., Tsanakas, P., and Hatzigeorgiou, A. G. (2009) DIANA-microT web server. Elucidating microRNA functions through target prediction. Nucleic Acids Res. 37, W273–276

16. Natarajan, R., Gonzales, N., Lanting, L., and Nadler, J. (1994) Role of the lipoygenase pathway in angiotension II-induced vascular smooth muscle cell hypertrophy. Hypertension 23, 1142–1147

17. Liu, X., Cheng, Y., Chen, X., Yang, J., Xu, L., and Zhang, C. (2011) MicroRNA-132-mediates Ang II-induced miR-132 in VSMC. Circ. Res. 109, 835–840

18. Delphin, E. S., and Zhang, C. (2009) MicroRNA-145, a novel smooth muscle tumor suppressor protein. J. Biol. Chem. 284, 16426–16431

19. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M., and Sellers, W. R. (1999) Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol-3-kinase/Akt pathway. Proc. Natl. Acad. Sci. U.S.A. 96, 1110–1115
41. Shaked, I., Meerson, A., Wolf, Y., Avni, R., Greenberg, D., Gilboa-Geffen, A., and Soreq, H. (2009) MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity 31, 965–973
42. Lagos, D., Pollara, G., Henderson, S., Gratrix, F., Fabani, M., Milne, R. S., Gotch, F., and Boshoff, C. (2010) miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. Nat. Cell Biol. 12, 513–519
43. Strum, J. C., Johnson, J. H., Ward, J., Xie, H., Feild, J., Hester, A., Alford, A., and Waters, K. M. (2009) MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. Mol. Endocrinol. 23, 1876–1884
44. Viedt, C., Vogel, J., Athanasiou, T., Shen, W., Orth, S. R., Kübler, W., and Kreuzer, J. (2002) Monocyte chemoattractant protein-1 induces proliferation and interleukin-6 production in human smooth muscle cells by differential activation of nuclear factor-κB and activator protein-1. Arterioscler. Thromb. Vasc. Biol. 22, 914–920
45. Ma, J., Wang, Q., Fei, T., Han, J. D., and Chen, Y. G. (2007) MCP-1 mediates TGF-β-induced angiogenesis by stimulating vascular smooth muscle cell migration. Blood 109, 987–994
46. Daugherty, A., Manning, M. W., and Cassis, L. A. (2000) Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J. Clin. Invest. 105, 1605–1612
47. Hernández-Presa, M., Bustos, C., Ortego, M., Tuñon, J., Renedo, G., Ruiz-Ortega, M., and Egidio, J. (1997) Angiotensin-converting enzyme inhibition prevents arterial nuclear factor-κB activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early accelerated atherosclerosis. Circulation 95, 1532–1541
48. Nemenoff, R. A., Simpson, P. A., Furgeson, S. B., Kaplan-Albuquerque, N., Crossno, J., Garl, P. J., Cooper, J., and Weiser-Evans, M. C. (2008) Targeted deletion of PTEN in smooth muscle cells results in vascular remodeling and recruitment of progenitor cells through induction of stromal cell-derived factor-1α. Circ. Res. 102, 1036–1045
49. Griengl, K. K., Minieri, C. A., Ollerenshaw, J. D., and Alexander, R. W. (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ. Res. 74, 1141–1148
50. Ichiki, T., Tokunou, T., Fukuyama, K., Iino, N., Masuda, S., and Takeshita, A. (2003) Cyclic AMP response element-binding protein mediates reactive oxygen species-induced c-fos expression. Hypertension 42, 177–183
51. Lin, Y., Liu, X., Cheng, Y., Yang, J., Hao, Y., and Zhang, C. (2009) Involvement of microRNAs in hydrogen peroxide-mediated gene regulation and cellular injury response in vascular smooth muscle cells. J. Biol. Chem. 284, 7903–7913
52. Littlewood, T. D., and Bennett, M. R. (2007) Foxing smooth muscle cells. FOXO3a-CYR61 connection. Circ. Res. 100, 302–304
53. Mejlvang, J., Kristjansdottir, H., Vandewalle, C., Chernova, T., Sayan, A. E., Berx, G., Mellon, J. K., and Tulchinsky, E. (2007) Direct repression of cyclin D1 by SIP1 attenuates cell cycle progression in cells undergoing an epithelial mesenchymal transition. Mol. Biol. Cell 18, 4615–4624
54. Panizzi, J. R., Jessen, J. R., Drummond, I. A., and Solinca-Krezel, L. (2007) New functions for a vertebrate Rho guanine nucleotide exchange factor in ciliated epithelia. Development 134, 921–931
55. Hur, W., Rhim, H., Jung, C. K., Kim, J. D., Bae, S. H., Jang, W. Y., Yang, J. M., Oh, S. T., Kim, D. G., Wang, H. J., Lee, S. B., and Yoon, S. K. (2010) SOX4 overexpression regulates the p53-mediated apoptosis in hepatocellular carcinoma. Clinical implication and functional analysis in vitro. Carcinogenesis 31, 1298–1307
56. Kligys, K., Claiborne, J. N., DeBiase, P. J., Hopkinson, S. B., Wu, Y., Mizuno, K., and Jones, J. C. (2007) The slingshot family of phosphatases mediates Rac1 regulation of cofilin phosphorylation, laminin-332 organization, and motility behavior of keratinocytes. J. Biol. Chem. 282, 32520–32528
57. Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57