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miR-92a inhibits vascular smooth muscle cell apoptosis: role of the MKK4–JNK pathway

Lan Zhang,
Department of Vascular Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China

Department of Medicine, Vascular Biology Center, Medical College of Georgia, Georgia Regents University, 1459 Laney Walker Blvd, Augusta, GA 30912, USA

Mi Zhou,
Department of Cardiac Surgery, Rui Jin Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200025, China

Yingjie Wang,
Internal Medicine of Traditional Chinese Medicine, Shuguang Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

Weibin Huang,
Department of Vascular Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China

Gangjian Qin,
Department of Medicine-Cardiology, Feinberg Cardiovascular Research Institute, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

Neal L. Weintraub, and
Department of Medicine, Vascular Biology Center, Medical College of Georgia, Georgia Regents University, 1459 Laney Walker Blvd, Augusta, GA 30912, USA

Yaoliang Tang
Department of Medicine, Vascular Biology Center, Medical College of Georgia, Georgia Regents University, 1459 Laney Walker Blvd, Augusta, GA 30912, USA

Yaoliang Tang: yaotang@gru.edu

Abstract

Vascular smooth muscle cell (VSMC) apoptosis plays an important role in vascular remodeling and atherosclerotic plaque instability. Oxidative stress in diseased vessels promotes VSMC apoptosis in part by activating the c-Jun N-terminal kinase (JNK) pathway, which has been

Correspondence to: Yaoliang Tang, yaotang@gru.edu.

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identified as a molecular target of miR-92a in macrophages. Here, we examined the expression and biological activity of miR-92a in VSMC. Quiescent VSMC exhibited a low basal expression of miR-92a, which was positively regulated by serum stimulation and negatively regulated by H_2O_2. Overexpression of miR-92a decreased H_2O_2-induced VSMC apoptosis as indicated by TUNEL assay and cleaved caspase-3 protein levels. Using 3′UTRreporter assay, we found that miR-92a overexpression led to suppression of both mitogen-activated protein kinase kinase 4 (MKK4)- and JNK1-dependent luciferase activity. We also found that 10 mer seed match between miRNA: mRNA pair is more efficient than 8 mer seed match for us to identify authentic miRNA target. Protein levels of active phospho-JNK and phospho-c-Jun, downstream targets of the MKK4–JNK1 pathway, were also decreased by overexpressing miR-92a in VSMC under oxidative stress. Consistent with these findings, overexpression of MKK4 reversed the anti-apoptotic effects of miR-92a in oxidatively stressed VSMC. In conclusion, miR-92a overexpression inhibits H_2O_2-induced VSMC apoptosis by directly targeting the MKK4–JNK1 pathway.

Keywords

Vascular smooth muscle cells; miR-92a; JNK; Apoptosis; Oxidative stress

Introduction

Vascular smooth muscle cell (VSMC) apoptosis plays an important role in vascular remodeling and in the pathophysiology of diseases such as aneurysms, post-angioplastyrestenosis and atherosclerosis. For example, VSMC apoptosis alone is sufficient to induce features of vulnerability leading to plaque rupture [1]. Apoptosis of VSMC is thought to be triggered by oxidative stress present in the diseased vascular milieu [2]. However, controlling oxidative stress clinically is a challenging task and has thus far not been demonstrated to improve outcomes in patients with cardiovascular disease [3]. Identifying the mechanisms that regulate oxidative stress-induced VSMC apoptosis potentially may lead to novel therapeutic approaches that are more efficacious for these patients.

The c-Jun N-terminal kinase (JNK) pathway plays an important role in VSMC apoptosis induced by oxidative stress in the setting of atherosclerotic plaque instability and rupture [2]. In many cell types, JNK is activated by a cell signaling cascade involving upstream mitogen-activated protein kinases (MAPKs). Murakami et al. [4] demonstrated that hydrogen peroxide (H_2O_2), which is produced by both vascular and inflammatory cells, increased vascular cell apoptosis through JNK activation. Genetic ablation of JNK1 markedly protected cells against UV-induced apoptosis, and JNK1 knockout mice were protected against cardiac ischemia–reperfusion injury in vivo [5]. The JNK pathway can thus be considered a molecular linkage between oxidative stress and cellular apoptosis [6].

MicroRNAs (miRNAs) are a recently-discovered family of endogenous, ≈ 22 nucleotide regulatory RNAs that post-transcriptionally regulate gene expression [7]. Mature miRNAs can mediate translational repression by forming miRNA-induced silencing complexes,

which bind to the 3′-untranslated region (3′UTR) of target mRNAs [8]. MiRNAs have been reported previously to play important roles in regulating cell apoptosis [9–11] and the progression of vascular diseases [12, 13]. Amongst the various miRNAs, miR-92a is a component of the miR-17-92 cluster, which is highly expressed in young endothelial cells in comparison with senescent endothelial cells, which exhibit increased oxidative stress and apoptosis [14]. The role of miR-92a in regulating VSMC is unclear. In this regard, miR-92a expression was reported to be low in VSMC in comparison with endothelial cells [15]. However, VSMC exhibit marked phenotypic heterogeneity both in vivo and in vitro depending on their external environment, state of growth, sensitivity to oxidant stress, etc. [16].

Here, we undertook a detailed study to examine mir-92a expression and biological activity in VSMC. We observed that miR-92a expression in VSMC is low in the basal (quiescent) state but positively regulated by serum stimulation and negatively regulated by H_2O_2. Moreover, overexpressing miR-92a protects VSMC against apoptosis induced by H_2O_2-mediated oxidative stress. Mechanistically, we provide evidence that miR-92a targets the JNK pathway to modulate VSMC apoptosis.

Methods

Aortic VSMC isolation and culture

Vascular smooth muscle cell were isolated from the aortas of 2-month-old, male C57B/L6 mice (JAX Laboratory) using the medial explant method and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) as described previously [17]. Cells between passages 5–15 were used for subsequent studies. To assess miR-92a expression in VSMC in response to serum stimulation, VSMC were serum-starved overnight, and then exposed to DMEM containing 0 %FBS, 2 %FBS, 5 %FBS, 10 %FBS or 20 %FBS for 24 h. To determine the effects of H_2O_2 on miR-92a expression in VSMC, VSMC were treated with 100 μM H_2O_2 in DMEM containing 10 %FBS for 24 h. To evaluate apoptosis induced by H_2O_2, miRNA mimic or inhibitor treated VSMC were treated with 100 lM H_2O_2 in DMEM with 10 %FBS for 16 h. ForMKK4 overexpression experiments, VSMC were transfected with pCDNA3 Flag- MKK4 (Addgene 14615) plasmid [18].

Synthetic miRNA transfection

For miRNA overexpression experiments, 100 nM double-stranded miR-92a mimic (GenePharma, China) or control miRNA (control) in 100 μL "R buffer" was transfected into VSMC using the Neon electroporation transfection system (Invitrogen). Settings were optimized at 1,400 V with two 20 ms pulses. For miRNA anti-sense inhibitor experiments, 100 nM single-stranded 2′O-methyl enhanced miR-92a or control inhibitor (GenePharma, China) was transfected into VSMC using Neon transfection system.

Quantitative reverse transcription polymerase chain reaction (QRT-PCR)

Total RNA from VSMC was extracted by RNAzol RT (Molecular Research Center, Inc. Cincinnati, OH) following the manufacturer's instructions. Isolated RNAs were

polyadenylated using the Ncode miRNA first-strand cDNA synthesis kit (Invitrogen). The cDNA synthesized was used to perform quantitative PCR on an M × 3000P Real-Time PCR System (Agilent Technologies, Santa Clara, CA) using the SensiMix SYBR kit (Bioline, Tauton, MA). Amplification was performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Protein extraction and western blot analysis

Cells were lysed in RIPA buffer (50 mMTris–HCl, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, PH 8.0) supplemented with a protease inhibitor cocktail (Roche Applied Science). The protein concentration was measured using a Bradford protein assay kit (Coomassie Plus Protein Assay reagent, Thermo). Protein samples were separated by 10 % SDS-PAGE (Bio-Rad) and electroblotted onto 0.45 μm Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 5 % Blotting-Grade Blocker (Bio-Rad) in PBST for 1 h at room temperature and incubated overnight at 4 °C with the respective antibodies: rabbit anti-MKK4, rabbit anti-JNK1, rabbit anti-phosphate JNK1, and rabbit anti-phosphate c-Jun (1:1,000; Cell Signaling, Danvers, MA); mouse anti-GAPDH antibody (1:4,000; Millipore, Billerica, MA); or rabbit anti-cleaved caspase 3 (1:500, Sigma-Aldrich, St. Louis, MO). Then, membranes were incubated for 1 h at room temperature with Amersham ECL peroxidase-linked secondary antibodies: sheep anti-mouse IgG (1:10,000, GE Healthcare) or donkey anti-rabbit IgG (1:10,000, GE Healthcare). Western blot immunoreactivity was detected using a Super Signal West Femto Maximum Sensitivity Substrate Kit (Thermo) with a C-DiGit Blot Scanner (LICOR Biosciences, Lincoln, Nebraska).

Dual luciferase reporter assay

Luciferase constructs were made by ligating oligonucleotides containing the putative target site of the wild-type and mutated mouse mRNA 3′UTR into the Pme1 and Xba1 site of the pmirGLO luciferase reporter vector (Promega, Madison, WI). The new vectors were termed pGLOmir-gene-wt and pGLOmir-gene-mut, respectively. The oligo sequences used in the aforementioned constructs are listed in Table 1.

For reporter assays, miR-92a or control mimic treated VSMC were transfected with pGLOmir-gene wt or pGLOmir-gene mut plasmids using Neon transfection system as described above. Luciferase activity was measured 24 h after transfection using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocols. Relative luciferase activity was calculated by normalizing the firefly luminescence (Fluc) to the renilla luminescence (Rluc); the ratio of Fluc to Rluc activity in control mimic treated VSMC was set to 1.

Immunofluorescence and confocal microscopy

For cell staining, cells were plated on 8-well chamber slides (Millipore, Billerica, MA) and subjected to H_2O_2 treatment as described above. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end-labeling (TUNEL) staining for apoptotic nuclei was performed using DEAD End TUNEL kit (Promega, Madison, WI) according to the manufacturer’s instructions with minor modifications. Briefly, cells were fixed in 4 %

paraformaldehyde for 25 min and then treated with permeabilization solution (0.2 % Triton X-100 solution in PBS) for 5 min at room temperature. Labeling reactions were performed with 100 μL of reaction buffer for 60 min at 37 °C in a humidified chamber, followed by steptavidin Alexa Fluor 488 or 555 conjugate (1:400, Life Technologies, Carlsbad, CA) staining. Slides were mounted using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and imaged using a Zeiss 510 Laser Scanning Microscope (Carl Zeiss, Thornwood, NY). Apoptosis was evaluated as the average number of TUNEL-positive cells per DAPI labeled cells at high-power magnification (×250).

Statistical analysis

Results are presented as mean ± SEM. Comparisons between groups were made by one-way analysis of variance or two-tailed Student's t test. Differences were considered statistically significant at $p < 0.05$.

Results

miR-92a expression in VSMC

To examine miR-92a expression under different levels of stimulation, VSMC were placed in culture media supplemented with 0, 2, 5, 10 or 20 % FBS for 24 h. RT-PCR analysis demonstrated that increasing concentrations of FBS were associated with increased expression of miR-92a, suggesting that miR-92a expression in VSMC is upregulated by growth factors present in FBS (Fig. 1a–c). To evaluate the effects of H_2O_2 on miR-92a expression in VSMC, we treated VSMC with H_2O_2 (100 μm) for 24 h. RT-PCR analysis demonstrated that miR-92a expression was significantly reduced in H_2O_2-treated VSMC compared with control cells (Fig. 1d, e), suggesting that H_2O_2-mediated oxidative stress inhibits miR-92a expression in VSMC.

miR-92a overexpression inhibits VSMC apoptosis induced by oxidative stress

To investigate the effects of overexpression of miR-92a on VSMC apoptosis under oxidative stress, we transfected a double-stranded miR-92a mimic into VSMC, which reduced H_2O_2-induced TUNEL (+) VSMC by ~ 40 % compared with the control mimic (Fig. 2a, b). Moreover, Western blot analysis showed that the miR-92a mimic reduced cleaved caspase-3 protein levels after 16 h of H_2O_2 oxidative stress (Fig. 2c, d).

To evaluate the specificity of miR-92 overexpression, VSMC were co-transfected with miR-92a mimic plus antimiR- 92a or a control miR. Two days later, transfected VSMC were exposed to 100 lm H_2O_2 for 16 h. As shown in Fig. 3, anti-miR-92a completely abrogated the protective effects of miR-92a overexpression against VSMC apoptosis. Taken together, these data demonstrate miR-92a overexpression inhibits VSMC apoptosis induced by H_2O_2.

MKK4 and JNK1 are direct targets of miR-92a

Mitogen-activated protein kinase (MAPK) kinase 4 has been reported to directly phosphorylate and activate JNK in response to cellular stress and pro-inflammatory cytokines [19]. Although miR-92a has been reported to modulate MKK4 expression in endothelial cells [15, 20], MKK4 has not been identified as a direct target of miR-92a in

VSMC. We performed bioinformatics algorithm analysis via TargetScan, which identified two conserved sites on the murine MKK4 3′UTR (NM_009157) and one conserved site on murine JNK1 3′UTR (NM_016700) as potential miR-92a targets. The sequence of miR-92a and its predicted binding sites on mouse MKK4 and JNK1 3′UTR are shown in Fig. 4a, d. To obtain evidence that MKK4 and JNK1 are direct targets of miR-92a, luciferase reporter constructs were generated by cloning either the wild-type (wt) 3′-UTR or a mutated (mut) portion of the 3′-UTR of MKK4 and JNK1 into the pmirGLO vector. Transfection with these vectors demonstrated that miR-92a overexpression significantly reduced the luciferase activities of the wild-type MKK4 (site2) and JNK1 reporters, but not the mutant constructs($p < 0.05$), confirming that the target site directly mediates repression of luciferase activity through seed-specific binding (Fig. 4c, e). In contrast, miR-92a overexpression did not significantly reduce the luciferase activity of the wild-type MKK4 construct (site1) (Fig. 4b). This observation differs from a recent report in macrophages demonstrating that miR-92a interacts with both predicted sites on MKK4 [21].

miR-92a regulates the MKK4-JNK1 pathway in oxidatively stressed VSMC

Since JNK1 pathway is involved in VSMC apoptosis induced by oxidative stress [22], and both MKK4 and JNK1 were identified as target genes for miR-92a, we investigated whether miR-92a regulates their expression in H_2O_2-treated VSMC. We observed that overexpression of miR-92a reduced the level of MKK4 protein by ~ 30 % (Fig. 5a), and p54 JNK1 protein by ~ 20 % (Fig. 5b), in H_2O_2-treated VSMC; this reduction of MKK4 and JNK1 lead to attenuation of both p54 and p46 JNK activation (Fig. 5c), and a significant decrease in the level of phospho- c-Jun (Fig. 5c), downstream targets of the MKK4– JNK1 pathway. These data suggest that MKK4 and JNK1 are down-regulated by miR-92a to inhibit VSMC apoptosis induced by oxidative stress.

To establish a mechanistic link between MKK4 down-regulation and suppression of H_2O_2-induced VSMC apoptosis by miR-92a, we overexpressed MKK4 by plasmid transfection. As expected, overexpression of MKK4 significantly increased the number of TUNEL(+) VSMC compared to GFP control (Fig. 6a, b), suggesting that MKK4 overexpression sensitizes VSMC to H_2O_2- induced apoptosis. Importantly, overexpression of MKK4 blocked the protective effects of miR-92a against VSMC apoptosis (Fig. 6c, d). The results from these experiments are summarized in Fig. 6e, and a schematic diagram depicting mechanisms whereby miR-92a inhibits H_2O_2- induced VSMC is shown in Fig. 6f.

Discussion

In this study, we provide evidence that miR-92a is expressed in serum-stimulated VSMC, and its expression is inhibited by oxidative stress induced by H_2O_2. Overexpression of miR-92a protects VSMC against H_2O_2-induced apoptosis, most likely by inhibiting the MKK4-JNK signaling pathway. We further demonstrate that both MKK4 and JNK1 are direct gene targets of miR-92a in VSMC.

miR-92a, a member of miR17-92 cluster, is reported to be highly expressed in endothelial cells. Down-regulation of miR-92a expression has been reported in the context of oxidative stress and aging. Chaudhry et al. [23] demonstrated that radiation-induced oxidative stress

represses miR-92a expression in human lymphoblast cells. Rippe et al. [14] reported that senescence of human endothelial cells is associated with reduced expression of miR-92a and enhanced apoptosis. Similar findings were reported by Ohyashiki et al. [24] in human lymphocytes. Conversely, miR-92a expression was reported to be very low in VSMC [15], suggesting that it may not play an important role in regulating apoptosis in this cell type. While we also observed that the basal expression of miR-92a was very low in serum-starved (quiescent) VSMC, miR-92a expression was up-regulated by serum stimulation, and its expression was attenuated by H_2O_2-induced oxidative stress. We also found that serum-stimulation protects VSMC against H_2O_2-induced apoptosis (Suppl Fig. 1), however, this protective effect might not be contributed to miR-92a alone, and many growth factors in FBS, such as VEGF, can activate Akt signaling, a key survival pathway, and protect VSMC against apoptosis induced by oxidative stress.

Fetal bovine serum contains many growth factors needed to support cell proliferation, including VEGF, epidermal growth factor (EGF), and PDGF [25]. These growth factors activate key transcription factors which, in turn, lead to upregulation of miRNAs. The VEGF-, EGF- and PDGF-responsive transcription factors, including E2F3 and Pim-1, have been reported to positively regulate miR17-92 expression [26–28]. The promoter regions of miR17-92 genes contain an E2F binding site (TTTSSCGC), and binding of E2F3 to these promoter regions activates their expression and promotes cell proliferation [27, 28]. Thomas et al. [26] reported that the proto-oncogene Pim-1 is part of the network that regulates transcription of the miR-17-92 cluster. Therefore, the transcriptional program activated by serum to promote proliferation may at the same time inhibit apoptosis through a miR-92a-dependent mechanism.

Our findings confirm a recent study which also used a luciferase reporter assay to show that miR-92a regulates expression of MKK4 by targeting its 3′UTR [21]. However, our results differ in that we were only able to confirm that one of the predicted binding sites (site 2) is an authentic miR-92a target. One possible explanation for this discrepancy is that there is a 10 nucleotide match between the sequence of site 2 (503–510) of the 3′UTR and miR- 92a, while there is only an 8 nucleotide match with the sequence of site 1 (105–112). In addition, Lai et al. [21] cloned wild-type MKK4 3′UTR, which included both binding sites, into a single luciferase reporter plasmid, whereas we constructed separate plasmids for each binding sequence. Finally, it is noteworthy that our study was conducted in VSMC, and the study by Lai et al. in macrophages.

Our findings pertaining to miR-92a expression and apoptosis of VSMC under oxidative stress are also consistent with prior reports in other cell types. Ohyashiki et al. [29] demonstrated that anti-miR-92a-treated human myeloid and lymphoid cells exhibited increased apoptosis. Moreover, miR-92a inhibition was reported to induce apoptosis of human glioma cells via directly targeting the Bim gene. The signaling mechanisms whereby miR-92a regulates oxidative stress-mediated apoptosis are poorly understood. Our data point towards a role for miR-92a in regulating the JNK pathway, an important mediator of stress-induced cell apoptosis [30–32]. This pathway is triggered by activation of the mitogen-activated protein kinase family (MAP3Ks), e.g. MEKK, which then activates MAP2K, e.g. MKK4 and MKK7 [33]. MKK4 is able to activate both the JNKs as well as p38 MAPKs,

whereas MKK7 specifically activates JNKs [34]. Stress-induced JNK pathway activation increases the expression of pro-apoptotic proteins and promotes apoptosis via p53/73-and/or c-Jun/AP1 dependent mechanisms [35–37]. JNK can also initiate ischemia- and TNF alpha-induced apoptosis via promoting the release of Smac/Diablo [38, 39]. Moreover, JNK activation phosphorylates Bcl2 protein to antagonize its anti-apoptotic activity [30]. Iaconetti et al. [15] reported that miR-92a inhibition increases phosphorylated JNK, and that miR-92a modulates MKK4 expression, in endothelial cells. A reciprocal relationship between miR-92a and activation of the JNK/c-Jun pathway, and production of inflammatory cytokines, was also reported in macrophages [21]. In this study, we observed thatMKK4and JNK1 protein levels were markedly decreased by miR-92a overexpression in VSMC. We further demonstrated that both MKK4and JNK1 are directly targeted by miR-92a in VSMC. In addition, overexpression of MKK4 abrogated the antiapoptotic effects of miR-92a in VSMC under oxidative stress. These findings suggest that miR-92a has anti-apoptotic effects in oxidatively stressed VSMC, which may partly be attributed to the suppression of JNK pathway.

In conclusion, we report that expression of miR-92a in VSMC is regulated by growth factors and oxidative stress. MiR-92a may play a functional role in VSMC by protecting against oxidative stress-induced apoptosis, at least in part through the inhibition of MKK4/JNK1 signaling. These findings may be useful to direct miRNA-based therapeutic strategies aiming to enhance protection of VSMC under oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

VSMC	Vascular smooth muscle cells
MAPK	Mitogen-activated protein kinase
MKK4	Mitogen-activated protein kinase (MAPK) kinase 4
JNK	C-Jun N-terminal kinase

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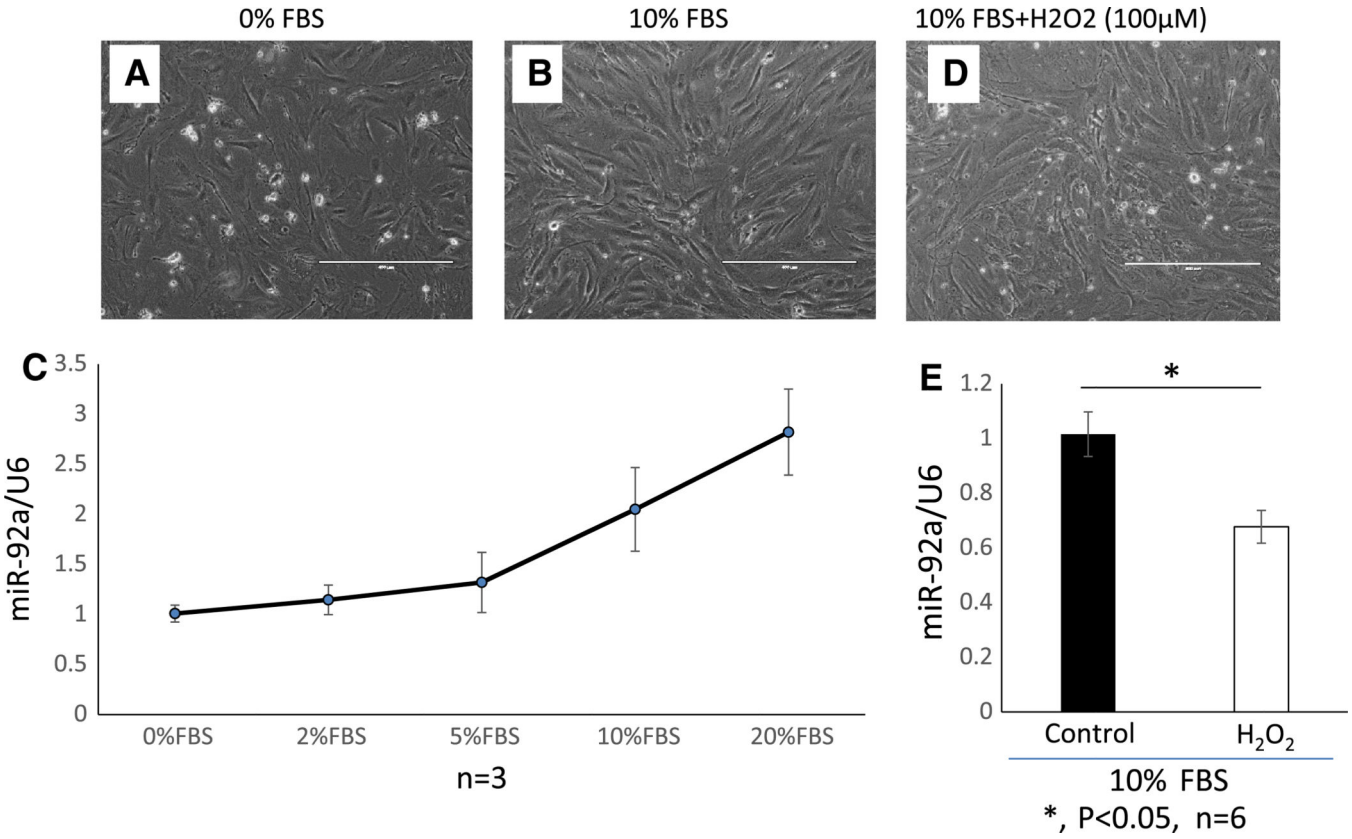

Figure 1.

a, **b** Morphology of mouse aortic VSMC in culture under 0 and 10 % FBS; **c** VSMC were maintained in DMEM with 0, 2, 5, 10 or 20 % FBS for 24 h. Quantitative RT-PCR showed that miR-92a expression in VSMC was upregulated by serum in a dose dependent manner, n = 3; **d** morphology of mouse VSMC in culture under 10 % FBS and treated with 100 lM H_2O_2 for 24 h; **e** quantitative RT-PCR showed that miR-92a expression in VSMC was downregulated by H_2O_2- mediated oxidative stress. *$p < 0.05$, n = 6

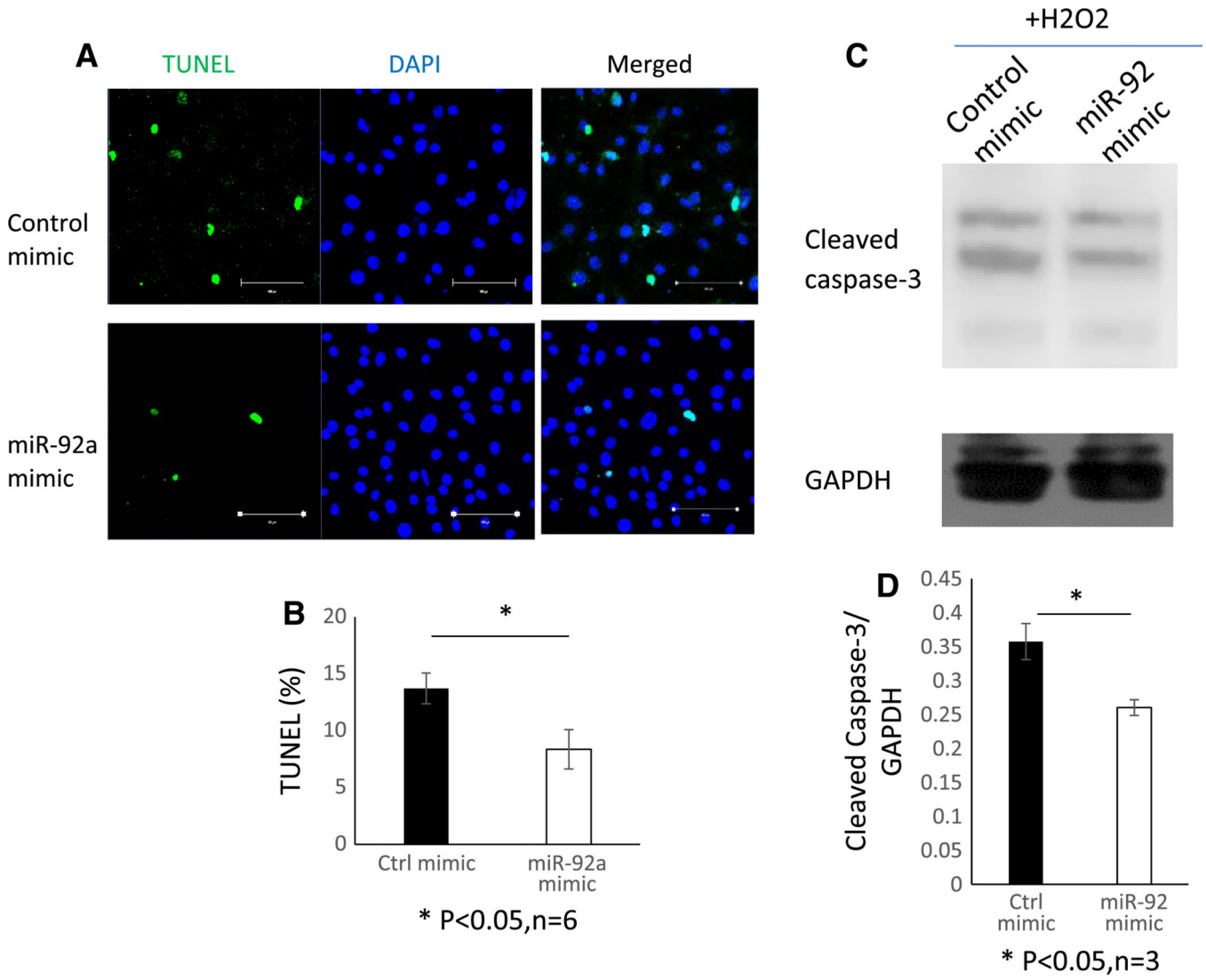

Figure 2.

Anti-apoptotic effects of miR-92a on VSMC under oxidative stress (100 μM H_2O_2 for 16 h): **a**, **b** TUNEL staining (*green*) showing representative images (**a**) and quantitative data (**b**); nuclei are stained with DAPI; **c** Western blot showing cleaved caspase 3 protein levels in oxidatively-stressed VSMC; representative blot is shown in (**c**) and quantitative data in (**d**) (Color figure online)

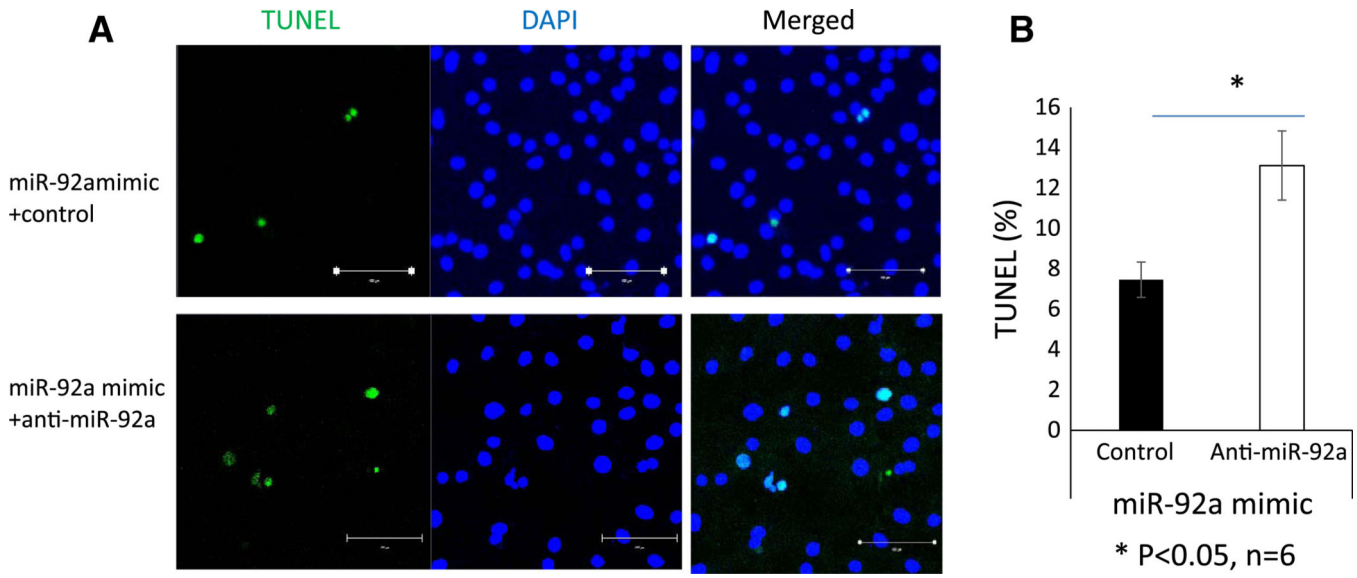

Figure 3.

Anti-miR-92a abrogates the protective effects of miR-92a against VSMC apoptosis under oxidative stress (100 μM H_2O_2 for 16 h). **a**, **b** TUNEL staining (*green*) showing representative images (**a**) and quantitative data (**b**); nuclei are stained with DAPI (Color figure online)

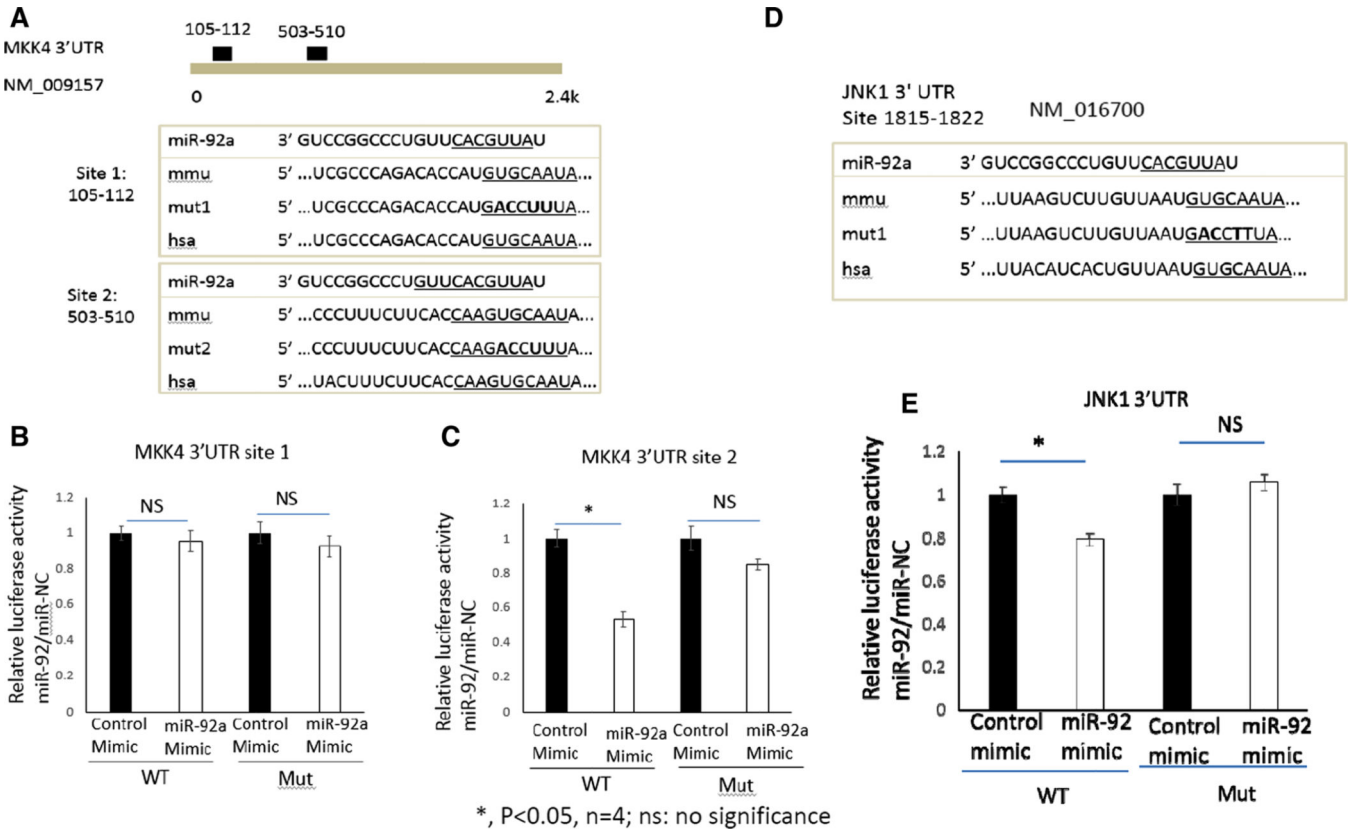

Figure 4.

Confirmation of target genes of miR-92a in VSMC. **a** The wild-type (WT) and mutated (MUT) 3′UTR of mouse MKK4, with the conserved seed region (*underlined*) and base substitutions (*bold*) shown; **b**, **c** effects of miR-92a overexpression on luciferase activities of wild-type and mutant MKK4 site1 and site2 reporter constructs; **d** the WT and MUT 3′UTR of mouse JNK1, with the conserved seed region and base substitutions shown; **e** effects of miR-92a overexpression on luciferase activities of the wild-type and mutant JNK1 reporter constructs. *$p < 0.05$, n = 4

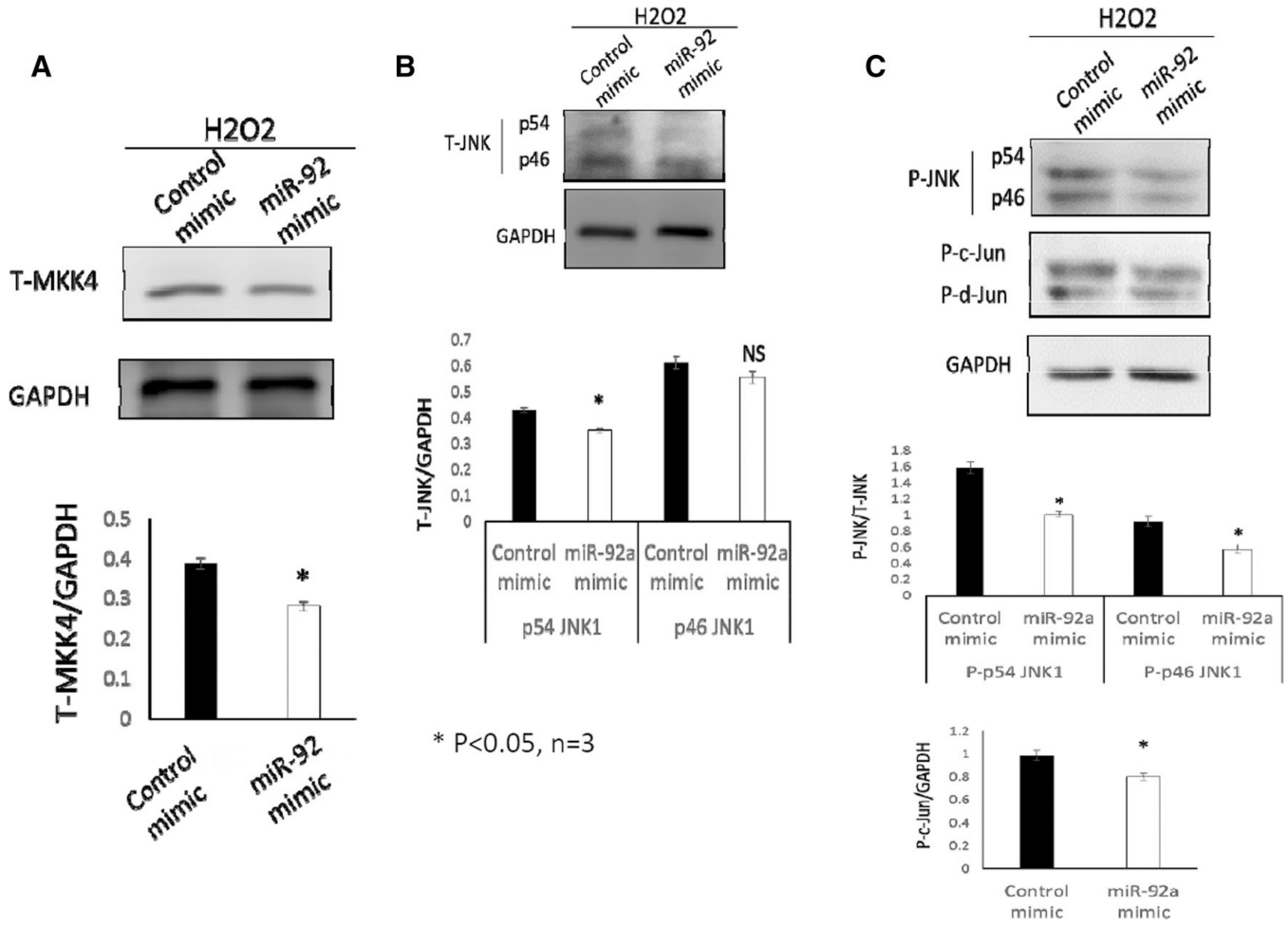

Figure 5.

Overexpression of miR-92a regulates the MKK4-JNK pathway in VSMC under oxidative stress. **a–c** Western blots (representative blots and quantitative data) show the effects of miR-92a overexpression on MKK4, JNK1, phos-JNK1 and phos-c-Jun in VSMC treated with H_2O_2, *$p < 0.05$, n = 3

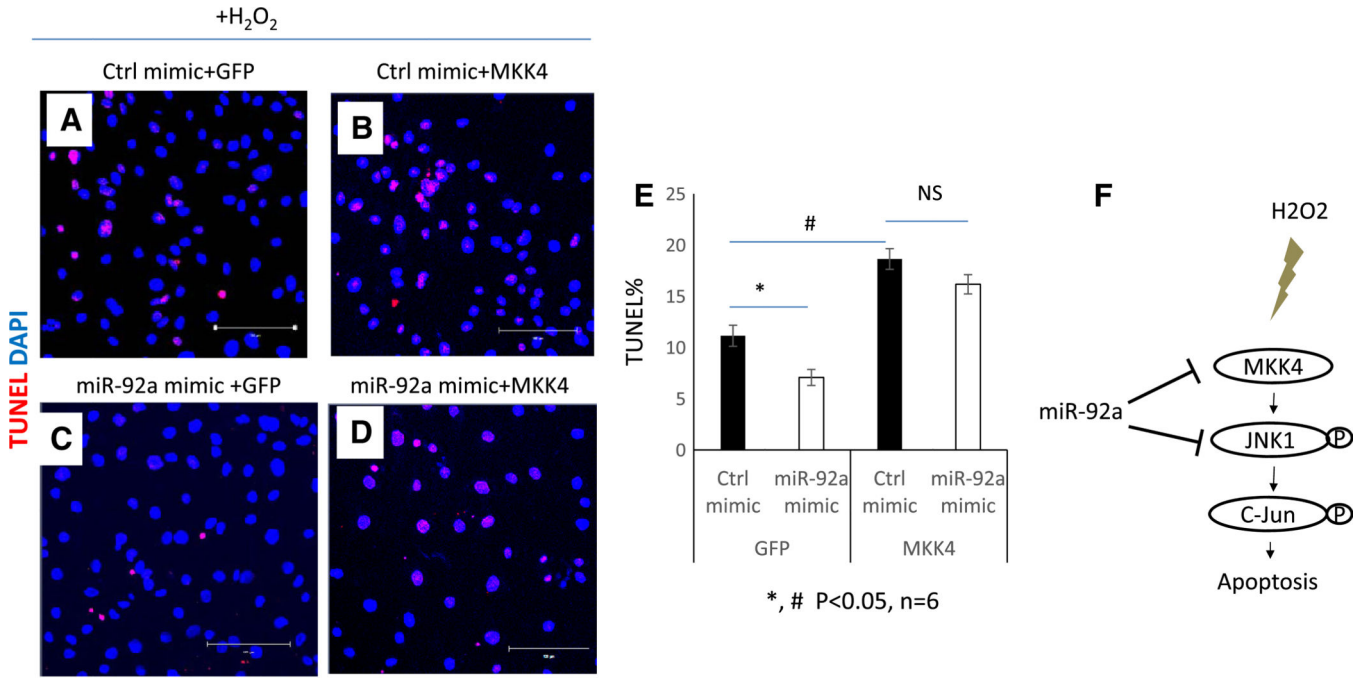

Figure 6.

Overexpression of MKK4 using pCDNA3 Flag-MKK4 plasmid abrogates the anti-apoptotic effects of miR-92a in VSMC. **a–d** VSMC were transfected with MKK4 or GFP control vector and subjected to oxidative stress (100 μM H_2O_2 for 16 h). **a–d** Representative images of apoptotic VSMC stained with TUNEL (*red*) and counterstained with DAPI (*blue*); **e** quantitative data showing percentage of TUNEL(+) VSMC. *$p < 0.05$, n = 6; **f** schematic diagram showing mechanisms whereby miR-92a inhibits H_2O_2-induced VSMC apoptosis (Color figure online)

Table 1

Primers used in 3′UTR studies

Oligos	Sequence5′–3′
MKK4 site 1 (NM_009157)	
MKK4 wt-sense	5′ AAAC TA GCGGCCGC TAGT TCGCCCAGACACCATGTGCAATAT 3′
MKK4 wt-antisense	5′ CTAGA TATTGCACATGGTGTCTGGGCGA ACTA GCGGCCGC TA GTTT3′
MKK4 mut-sense	5′ AAAC TA GCGGCCGC TAGT TCGCCCAGACACCATGACCTTTA T3′
MKK4 mut-antisense	5′ CTAGA TAAAGGTCATGGTGTCTGGGCGA ACTA GCGGCCGC TA GTTT3′
MKK4 site 2 (NM_009157)	
MKK4 wt-sense	5′ AAAC TA GCGGCCGC TAGT CCCTTTCTTCACCAAGTGCAATA T3′
MKK4 wt-antisense	5′ CTAGA TATTGCACTTGGTGAAGAAAGGG ACTA GCGGCCGC TA GTTT3′
MKK4 mut-sense	5′ AAAC TA GCGGCCGC TAGT CCCTTTCTTCACCAAGACCTTTA T3′
MKK4 mut-antisense	5′ CTAGA TAAAGGTCTTGGTGAAGAAAGGG ACTA GCGGCCGC TA GTTT3′
JNK1 (NM_016700)	
JNK1 wt-sense	5′ AAAC TA GCGGCCGC TAGT TTAAGTCTTGTTAATGTGCAATA T3′
JNK1 wt-antisense	5′ CTAGA TATTGCACATTAACAAGACTTAA ACTA GCGGCCGC TA GTTT3′
JNK1 mut-sense	5′ AAAC TA GCGGCCGC TAGT TTAAGTCTTGTTAATGACCTTTA T3′
JNK1 mut-antisense	5′ CTAGA TAAAGGTCATTAACAAGACTTAA ACTA GCGGCCGC TA GTTT3′