Curcumin inhibits proliferation, migration and neointimal formation of vascular smooth muscle via activating miR-22

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\textbf{ABSTRACT}

\textbf{Context:} Curcumin has antitumor, antioxidative, anti-inflammatory, and anti-proliferative properties.

\textbf{Objective:} To investigate the role of miR-22 during curcumin-induced changes in vascular smooth muscle cells (VSMC) and neointima formation in balloon-injured rat abdominal aorta.

\textbf{Materials and methods:} Sprague-Dawley rats were randomised to the sham-operated (n = 10), operated control (injured, n = 10), and curcumin treatment (n = 10) groups. miR-22 expression was determined by real-time PCR. SP1 was assessed by western blot and real-time PCR. Rat aortic smooth muscle A7r5 cells were used to determine VSMC proliferation and migration, which were measured by the MTS, EdU staining, Transwell, and wound healing assays.

\textbf{Results:} miR-22 levels declined following arterial balloon injury in vivo (48% at 3d, p < 0.05) and serum stimulation in vitro (45% at 24 h, p < 0.01). Functional studies revealed that miR-22 negatively regulated the proliferation and migration of VSMCs by directly targeting the SP1 transcription factor in VSMCs. Curcumin increased the expression of miR-22 (81%, p < 0.05) and decreased the protein expression of SP1 in VSMCs (25%, p < 0.05). miR-22 inhibition was found to attenuate the effects of curcumin on VSMC functions. Curcumin increased miR-22 (46%, p < 0.01), decreased the SP1 protein (19%, p < 0.05), and inhibited vascular neointimal area (48%, p < 0.01) in vivo.

\textbf{Discussion:} The miR-22/SP1 pathway is involved in the protective role of curcumin during arterial balloon injury, but the mechanisms remain unclear.

\textbf{Conclusion:} miR-22 is involved in the inhibitory effects of curcumin on VSMCs’ proliferation, migration and neointima hyperplasia after arterial balloon injury in rats. Curcumin could be used to prevent neointimal hyperplasia after angioplasty.

\textbf{Introduction}

During interventional therapy for coronary heart disease, balloon dilatation, and stent implantation can damage the vascular endothelium by mechanical force, causing smooth muscle cells of the middle layer to migrate and proliferate, eventually leading to vascular stenosis or stent restenosis (Mintz et al. 1996; Curcio et al. 2011). The vascular smooth muscle cells (VSMCs) can be controlled during this process through various environmental stimuli (Alexander and Owens 2012). VSMC-specific genes become differentially expressed as the smooth muscle phenotype switches. For example, smooth muscle \textalpha-actin (\textalpha-SMA) and smooth muscle calponin levels decrease in the proliferative phenotype and increase in the contractile phenotype (Regan et al. 2000; Cheng et al. 2009).

Recent studies have demonstrated that the expression profiles of several miRNAs including miR-143/145 (Cordes et al. 2009), miR-133 (Torella et al. 2011), miR-23 (Iacovetti et al. 2015), and miR-221/222 (Davis et al. 2009) are dysregulated during the proliferation and differentiation of VSMCs. The specific molecular mechanisms that govern VSMC proliferation and re-endothelialization after stenting are not completely understood.

miRNAs are a group of short non-coding RNA that have critical functions in a range of human diseases, including diabetes, cardiovascular disease, kidney disease, and cancer (Paul et al. 2018). miR-22 was the first discovered miRNA with antitumor properties (Pandey and Picard 2009). Its expression has been subsequently identified to be dysregulated in multiple types of cancer, including gastric, breast, and liver cancer (Xiong et al. 2010; Kong et al. 2014). Recent studies have also revealed that miR-22 is richly expressed in the heart (Hu et al. 2012), where it plays a role in vascular remodelling (Zheng and Xu 2014), cardiac hypertrophy (Gurha et al. 2012; Huang et al. 2013), and spontaneous hypertension (Friese et al. 2013). It remains unclear as to whether miR-22 regulates the VSMC phenotype and neointimal formation in injured vascular.

Curcumin is an active compound derived from turmeric (Curcuma longa Linn. Zingiberaceae) (Hewlings and Kalman 2018). Curcumin has antitumor, antioxidative, anti-inflammatory, and anti-proliferative properties.
Curcumin has been shown to display a variety of activities, including antioxidant, anti-inflammatory, antiproliferation, and anti-angiogenic properties (Maheshwari et al. 2006; Anand et al. 2007). Recent studies have unveiled that curcumin can inhibit balloon injury-induced VSMC migration and neointimal formation (Yang et al. 2006; Yu and Lin 2010; Sheu et al. 2013). Curcumin also prevents the development of hypertension in an AngII-induced hypertensive model (Yao et al. 2016). In addition, curcumin has been shown to modulate the expression profiles of many miRNAs including miR-15a, miR-22, miR-125, miR-146, miR-200, and miR-49, and has been proposed as a therapeutic compound that can improve cancer treatment and other therapies (Shishodia 2013; Teiten et al. 2013; Li et al. 2018).

This study aimed to better understand the role of curcumin in modulating VSMC proliferation and migration. Mechanistically, we show that miR-22 inhibits VSMC proliferation and migration in vitro by targeting the transcriptional factor SP1. We established, both in vitro and in vivo, that miR-22/SP1 mediates the inhibitory effects of curcumin on VSMC proliferation and migration.

**Material and methods**

**Drugs and reagents**

Dulbecco’s modified Eagle medium was purchased from Invitrogen (CA, USA). Curcumin was obtained from Sigma (c7727, St. Louis, MO, USA); foetal bovine serum (FBS) was purchased from Gibco (CA, USA). Overexpression or inhibition of miR-22 was performed through the transfection of miR-22 mimics or miR-22 inhibitors from RiboBio (Guangzhou, China), and X-tremeGENE siRNA was purchased from Roche (Basel, Switzerland). Cell proliferation assays were performed using the EdU Kit obtained from RiboBio (Guangzhou, China) and the MTS Kit purchased from Promega (WI, USA). Transwell chambers (8 μm) were purchased from Corning (NY, USA).

**Animals and artery balloon injury models**

All operations strictly adhered to the guidance of the Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (290–330 g) were purchased from the animal laboratory of the Health Science Centre of Southern Medical University. Thirty rats were randomised to the sham-operated group (sham, n = 10), operated but untreated control group (injured, n = 10), and curcumin treatment group (curcumin, n = 10). Balloon injury of the abdominal aorta was induced through the insertion of the deflated balloon through the left carotid artery down to the level of renal arteries. The rats were anaesthetised with 10% chloralhydraté (400 mg/kg, i.p.). The left carotid artery was catheterised with a 2-F deflated arterial balloon catheter that was placed distal to the renal arteries. Saline was injected into the tip of the anterior catheter to inflate the balloon, and the catheter was slowly pulled back to the diaphragm level. This procedure was repeated three times to ensure maximum removal of the vascular intima (Wang et al. 2004).

Curcumin was obtained from Sigma (c7727, St. Louis, MO, USA); foetal bovine serum (FBS) was purchased from Gibco (CA, USA). Overexpression or inhibition of miR-22 was performed through the transfection of miR-22 mimics or miR-22 inhibitors from RiboBio (Guangzhou, China), and X-tremeGENE siRNA was purchased from Roche (Basel, Switzerland). Cell proliferation assays were performed using the EdU Kit obtained from RiboBio (Guangzhou, China) and the MTS Kit purchased from Promega (WI, USA). Transwell chambers (8 μm) were purchased from Corning (NY, USA).

The rats in the curcumin group were given 50 mg/kg of curcumin (2.5 mg of curcumin was dissolved in 10 mL of DMSO and mixed with 190 mL of olive oil) by intraperitoneal injection once a day for 14 days. The rats in the sham and injured groups received intraperitoneal injections of the solvent. On days 3 or 14, the abdominal aorta was removed from sacrificed rats and placed in 4% paraformaldehyde (PFA) tubes for histological analysis or snap-frozen for RNA and protein extraction.

**Cell culture**

Rat aortic smooth muscle cells (A7r5) were purchased from the Shanghai Cell Bank (China). DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin were used to culture cells in a humidified environment in the presence of 5% CO2. The cells were plated at a density of 60%, and cultured for 12 h in serum-free DMEM. The cells were transfected with miR-22 mimics and/or miR-22 inhibitors for 6 h in serum-free DMEM, following which fresh DMEM containing 10% FBS was added. All the experiments were repeated three times independently.

**VSMC proliferation assays**

Proliferation assays were performed following the manufacturer’s protocols. For the EdU experiments, VSMC proliferation rates were calculated as the ratio of red-stained proliferating cells over the total cell number, assessed by fluorescence microscopy. For MTS assays, automatic measurements of the absorbance of cell samples were achieved by setting a threshold value of 490 nm (OD490) on a microplate reader. Absorbance values representative of cell proliferative capacity were compared between different treatment groups.

**VSMC migration assays**

Transwell invasion and scratch wound healing assays were used to determine cell migration. For scratch wound healing assays, transfected VSMCs were grown to 100% confluency in 6-well plates and serum-starved for 12 h. We created two straight-line scratches using a 200 μL pipette at the centre of the 6-well plate, which was washed with PBS to remove floating cells and cell debris. Cells were stimulated in DMEM plus serum for 24 h. The scratch boundaries were imaged under an inverted microscope and analysed using the Image J software. For the transwell migration assays, transfected VSMCs (5 × 104) were added to the upper chamber without FBS, and a medium containing 10% FBS was added to the lower chamber. Non-invasive cells were gently removed after 24 h. Cells invading the lower surface were fixed with 4% PFA, stained with gentian violet, and imaged on an inverted microscope. Cells that passed through the mesh were fixed with 4% PFA, and the invading cells were counted by microscopy following gentian violet staining.

**Dual-luciferase reporter assays**

Cells were seeded on 96-well plate; 24 h later, they were transfected with wild-type and mutated SP1 3’-UTR under the control of the pRL-CMV control vector (Promega, USA) and miR-22 mimics or miR-22 negative controls. Then, Firefly plasmid (0.2 μg), renilla plasmid (0.004 μg), and transfection reagent (0.25 μL) were mixed and incubated at room temperature for 5 min. microRNA and transfection reagents were mixed so that the final concentration of microRNA was 100 nM incubated at room temperature for 5 min. The transfection mix and microRNA mix were added to the cells. The cells were changed with fresh culture medium 6 h later. After 48 h, PBS-washed cells were lysed using a passive lysis buffer. The supernatants were mixed with LR II and read for luminescence values. The Stop &
Glo reagent was added, and the cells were read for luminescence values. Dual-luciferase reporter assays (Promega, USA) were used to evaluate the luciferase activity of the harvested cells.

RNA isolation and real-time PCR

For RNA extraction, the cells were lysed in Trizol reagent (Invitrogen, USA) and the SYBR Green Real-time PCR (Takara, China) assay was performed to detect mature miR-22 or SP1 mRNA expression. SYBR green Real-time PCR was performed using the following primers: Calponin: sense, 5′-ACCAAGCGGCAGTCTTTGGA-3′, antisense, 5′-CTGCCTTGTTGAGTGAATGG-3′; α-SMA: sense, 5′-CGGGTACTTCAGGGTACG-3′, antisense, 5′-CGGGTACTTCAGGGTACG-3′; SP1: sense, 5′-ACCTGGCAAGGCTGAGTGAATGG-3′, antisense, 5′-GAGTGGATATGTATGGGAGTGA-3′; GAPDH: sense, 5′-GCACCGTCAAGGCTGAGAAC-3′, antisense, 5′-GAGTGGATATGTATGGGAGTGA-3′; U6: sense, 5′-CTCGCTTCGGCAGCACA-3′, antisense, 5′-AACGCTTCACGATCTTTGAG-3′. U6 or GAPDH were used as endogenous controls. The 2^–ΔΔCT method was used for data processing. miR-22 mimic and miR-22 inhibitor were synthesised by Guangzhou RiboBio, and the sequences are as follows: MiR-22mimics sense: AAGCUGCCAGUUGAAGAACUGU; mimics antisense: AGUUCUUCACUGGCAGCUU; inhibitor: ACGAUUCUUCACUGGCAGCUU. The mimic NC and inhibitor NC sequences are proprietary (Guangzhou RiboBio).

Figure 1. MiR-22 expression in VSMCs and rat balloon injury model. (A) miR-22 levels at 24 h of starvation (T0), 3, 6, 12, 24, and 48 h (T3, T6, T12, T24, and T48) during active proliferation following 10% FBS stimulation. (B) Arteries were stained with H&E 14 days post-balloon injury in vivo (scale bar = 100 μm) (magnification of the original microscope image: 100×; the enlarged portion was enlarged digitally from the original high-resolution microscope image). (C) Intima transection area after the arteries were balloon-injured. (D) The neointimal/media (N/M) ratio after arteries were balloon-injured. (E) mRNA level of miR-22 in the sham group, day 3 and day 14 after the injury. (F-G) mRNA level of α-SMA (F) and calponin (G) in the sham group, day 3 and day 14 after the injury. ∗p < 0.05; ∗∗p < 0.01: vs. T0 or Sham group; #p < 0.05; ##p < 0.01: vs. injured-3d group. All values are expressed as the mean ± SD, n = 3 (A), n = 5 (B-G).
Western blot analysis

Cells were lysed in RIPA buffer (containing 0.1% PMSF), and the protein concentration of the cell lysates was assessed using BCA assays. Proteins were separated on SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in TBST and probed overnight with mouse polyclonal SP1 or GAPDH primary antibodies (Santa Cruz, 1:1000). Membranes were labeled with HRP-conjugated secondary antibodies (1:5000) for 2 h at room temperature and visualized using the enhanced chemiluminescence reaction ECL Kit (Beyotime, China).

Morphometric analysis for neointimal formation

Abdominal aortas were fixed in 4% PFA and subjected to paraffin embedding and sectioning for H&E staining. Sections (5-μm thick) were obtained at 100-μm intervals and mounted onto glass slides. Slides were H&E-stained to visualize vascular sections. Lumen, media, and neointimal were measured via computational image analysis (Image-Pro Plus).

EdU staining

Cells were cultured in the EdU solution for 2 h. Cells were treated by Immunol Staining Fix Solution and Enhanced Immunostaining Permeabilization Buffer (RiboBio). Cells were then stained by Apollo and Hoechst33342.

TUNEL staining

Cells were treated by Immunol Staining Fix Solution and Enhanced Immunostaining Permeabilization Buffer (Beyotime) and then treated with TUNEL detection buffer. Cells were analyzed by flow cytometry or microscopy.

Statistical analysis

All analyses were based on three independent experiments. Unpaired t-tests and one-way ANOVA tests with the LSD post hoc test were used to evaluate statistical significance. A p-value < 0.05 was deemed statistically significant.
Results

miR-22 expression during VSMC proliferation and in injured vascular walls

VSMCs were first starved with serum-free medium for 24 h and then treated with serum. miR-22 levels were detected by real-time PCR in rat serum-stimulated VSMCs (A7R5). We observed significant downregulation of miR-22 at 12 and 24 h after serum treatment (Figure 1(A)). At 48 h after serum stimulation, there was also a significant inhibition of miR-22 level in the cells, although it was much less than the inhibition that was observed at 12 and 24 h of stimulation. We next established the rat balloon injury model and observed increased neointimal area and neointimal/media ratio at 14 days after injury (Figure 1(B–D)). Furthermore, we assessed the levels of miR-22 in the rat balloon injury model and observed a decreased expression of miR-22 in the injury group at 3 days compared to the uninjured group, but increased expression of miR-22 at day 14 compared with day 3 (Figure 1(E)). α-SMA and calponin are well-established markers of VSMC differentiation. The expression of the two markers decreases as VSMCs proliferate. Interestingly, we observed a reduced expression of α-SMA and calponin at day 3 post the injury but increased expression at day 14 compared with day 3 (Figure 1(F–G)). Taken together, these data suggest that miR-22 expression is modulated during VSMC proliferation in vitro and in vivo.

miR-22 inhibits VSMC proliferation and migration

We then transfected VSMCs with the miR-22 mimic or used the miR-22 inhibitor to test whether miR-22 negatively regulates the ability of VSMCs to proliferate and migrate. The concentration–response analysis showed that 25 nmol/L miR-22 mimic was appropriate for 10% FBS stimulation (Figure S1A), while 50 nmol/L miR-22 inhibitor was the appropriate concentration following 2% FBS stimulation compared with 10% FBS (Figure S1B–C). As 10% FBS stimulation leads to decreased expression of miR-22 in VSMCs, exogenous inhibitors failed to induce a further decrease. Thus, 2% FBS was used thereafter. Interestingly, miR-22 mimic caused a significant decrease in the activity of VSMCs and reduced cell proliferation, as confirmed by MTS and EdU assays (Figure 2(A,C)). In contrast, miR-22 inhibition increased VSMC proliferation (Figure 2(B,D)). As expected, no significant differences were observed in VSMCs using mimic negative control (NC), inhibitor NC or NC transfection (Figure 2(A–D)). To assess the involvement of miR-22 during VSMC migration, wound healing (Figure 2(E–F)) and

Figure 3. MiR-22 downregulates the expression of SP1. (A) Potential miR-22 binding sequence of SP1 3’-UTR and mutation sites. mRNA (B) and protein level (C) of SP1 in control VSMCs, mimic NC-treated cells, and miR-22 mimic-treated cells. mRNA (D) and protein level (E) of SP1 in control VSMCs, inhibitor NC-treated cells, and miR-22 inhibitor-treated cells. (F) Luciferase activity of wild-type and mutant SP1 3’-UTR reporters were determined in HEK293T cells. *p < 0.05; **p < 0.01: vs. CTRL or WT-miR-22 NC group; All values are expressed as the mean ± SD, n = 3.
Transwell migration assays (Figure 2(G–H)) were performed. Consistent with the proliferation data, treatment of VSMCs with the miR-22 mimic reduced the cell migratory capacity in both assays, while treatment with the miR-22 inhibitor increased the migration (Figure 2(E–H)). No significant changes with the mimic NC or inhibitor NC in VSMCs were observed (Figure 2(E–H)).

miR-22 negatively regulates SP1 expression by targeting its 3′-UTR

Using the Pictar and TargetScan software from microRNA.org, we found that SP1 contains one putative miR-22 target site (Figure 3(A)). We, therefore, investigated the association between miR-22 and SP1 expression. miR-22 overexpression reduced SP1 expression under both mRNA and protein levels in VSMCs compared with the control cells (Figure 3(B–C)). By contrast, miR-22 inhibitor stimulation increased the mRNA and protein levels of SP1 (Figure 3(D–E)), confirming that miR-22 negatively regulates the expression of SP1. We confirmed these findings by transfecting miR-22 in HEK 293 T cells and showed that the luciferase activity of the wild-type SP1 3′-UTR was inhibited by the transfection (Figure 3(F)), the inhibition of which was rescued when the SP1 3′-UTR binding sites were mutated (Figure 3(F)). Taken together, these data demonstrate that miR-22 directly targets the SP1 3′-UTR and that SP1 is negatively regulated by miR-22 in VSMCs.

Curcumin inhibits the proliferation and migration of vascular smooth muscle by activating miR-22

We next examined the effects of curcumin on miR-22 in VSMCs. A concentration-response analysis showed that the best curcumin dose chosen for the upregulated expression of miR-22 was 10 μM (Figure S2A–B). We showed that curcumin increased miR-22 expression (Figure 4(A)), which could be counteracted by the miR-22 inhibitor (Figure 4(A)). As shown by MTS and EdU assays, curcumin led to a drastic reduction in VSMC proliferation, which could also be reversed by the miR-22 inhibitor (Figure 4(B)). To further assess the effects of curcumin during VSMC migration, we performed wound healing (Figure 4(E,F)) and transwell migration assays (Figure 4(G,H)). Consistent with the proliferation data, curcumin reduced the migratory capacity of VSMCs compared with untreated control cells, while the miR-
22 inhibitor partially rescued the inhibition (Figure 4(E,H)). These data show that curcumin inhibits VSMC proliferation and migration by miR-22.

**Curcumin increases α-SMA and calponin mRNA and decreases SP1 levels in VSMCs**

We then measured the expression of α-SMA and calponin in the VSMCs. Real-time PCR results revealed that miR-22 mimic treatment upregulated α-SMA and calponin mRNA, while their expression was downregulated by miR-22 inhibition (Figure 5(A,B)). Furthermore, α-SMA and calponin mRNA levels in VSMCs were significantly increased following curcumin treatment, which was rescued by the miR-22 inhibitor (Figure 5(C,D)). To further clarify the effects of curcumin on smooth muscle cells, we assessed the impact of curcumin on SP1 expression. Curcumin treatment decreased mRNA and protein expression of SP1 (Figure 5(E,F)), an effect that was partially reversed by miR-22 inhibitors (Figure 5(G–I)). Taken together, these data reveal that in VSMCs, curcumin increases α-SMA and calponin mRNA expression and decrease SP1 levels in a miR-22-dependent manner.

**Curcumin suppresses balloon injury-induced neointima formation**

We further determined whether curcumin could inhibit neointimal hyperplasia and modulate miR-22 in vivo. Furthermore, we showed that miR-22 levels were significantly decreased at day 3 post-injury (Figure 6(A)), and SP1 levels were increased on day 3 in response to vessel injury (Figure 6(B,C)), while curcumin treatment reversed both those effects (Figure 6(A,C)). mRNA levels of α-SMA and calponin were reduced at 3 days following the injury, and such reductions were also rescued by curcumin (Figure 6(D,E)). Neointima formation was examined by H&E staining at 14 days after the balloon injury. Importantly, at 14 days post-balloon injury, we found that curcumin treatment significantly reduced the neointimal area and neointimal/media ratio compared with the injured group (Figure 6(F–H)). Taken together, these results indicate that the anti-neointima formation effects of curcumin are mediated by miR-22/SP1 in response to vessel injury.

**Discussion**

In this study, we revealed that the levels of miR-22 decreased in proliferative VSMCs and in the vascular wall following injury. We demonstrated that treatment with a miR-22 mimic could partially inhibit VSMC functions such as proliferation and migration through targeting SP1 in vitro. Moreover, we showed that curcumin treatment increased miR-22 levels and reduced SP1 expression in VSMCs. miR-22 inhibitors reversed the inhibition of VSMC proliferation and migration by curcumin. We finally demonstrated that the miR-22/SP1 pathway is involved, at least in part, in the protective role of curcumin during vascular injury in vivo.

It is well known that miR-22 is ubiquitously expressed in a range of tissues (Neely et al. 2006). It was then shown that miR-22 participates in angiogenesis, age-associated vascular diseases, and cardiac hypertrophy (Gurha et al. 2012; Zheng and Xu 2014; Takeda et al. 2016). Nevertheless, whether miR-22 plays a role in the dedifferentiation of VSMCs was not established. Here, we confirmed that miR-22 could inhibit VSMC proliferation and migration. We further observed that the expression of miR-22
correlates with the expression of α-SMA and calponin. Upon vascular injury, SP1 binds to G/C-rich repressor elements and decreases SM gene expression (Wamhoff et al. 2004). Deaton et al. demonstrated that SP1 binding to the Kruppel-like factor 4 (KLF4) promoter increases in platelet-derived growth factor (PDGF)-BB-stimulated SMCs following aortic balloon injury in vivo (Deaton et al. 2009). SP1 encodes PU.1, a transcriptional factor of the ETS family, which can control the transcription of specific genes by itself or by interacting with other transcriptional factors such as NF-κB or C/EBPa (Rothenberg et al. 2019). Our data confirmed that miR-22 directly targets SP1 and suppresses the proliferation and migration of VSMCs. These results indicate the miR-22/SP1 axis functions as a novel regulatory signalling pathway during VSMC proliferation and migration. Recently, Yang et al. (2018) also reported similar data in which miR-22 was found to regulate the dedifferentiation of VSMCs and the formation of vascular neointima in mouse models. Huang et al. (2017) found that the expression of miR-22-3p decreases in arteriosclerosis obliterans arteries, indicating that miR-22-3p can regulate the proliferation, migration, and neointima formation by targeting HMGB1 in HASMC. The present study also provides evidence that curcumin can inhibit the proliferation and migration of VSMCs through miR-22.

Curcumin is a widely consumed polyphenol that has beneficial effects in cardiac disease by suppressing VSMC proliferation and neointima formation, and attenuating inflammation and oxidative stress (Jeong et al. 2012; Liu et al. 2012; Sheu et al. 2013; Yang et al. 2013). Curcumin has been shown to regulate the expression of multiple miRNAs in a variety of neoplastic and non-neoplastic diseases. Curcumin also regulates miRNA levels in pancreatic cells, increasing the expression of miRNA-22 and decreasing the expression of miRNA-199a (Sun et al. 2008). Moreover, curcumin exerts its effects on the miR-590-3p/CD40 axis to play a protective role in injured endothelial cells (Wu et al. 2017). In addition, Sheu et al. (2013) have shown that demethoxycurcumin can downregulate the FAK/PI3K/AKT axis

Figure 6. Protective role of curcumin in balloon injury models in vivo. mRNA expression of miR-22 (A) and protein levels (B-C) of SP1 in sham, injured, and curcumin-treated injured group 3 days post-balloon injury in vivo. mRNA expression of α-SMA (D) and calponin (E) in sham, injured, and curcumin-treated injured group 3 days post-balloon injury in vivo. (F) Representative images of H&E staining from sham, injured, and curcumin-treated injured group 14 days post-balloon injury in vivo. (scale bar = 100 μm) (G) Intima transection area and (H) neointimal/media (N/M) ratio after arteries were balloon-injured. *p < 0.05; **p < 0.01: vs. sham group; *p < 0.05; ***p < 0.01: vs. injured-3d or injured-14d group. All values are expressed as the mean ± SD, n = 5.
and suppress VSMC migration and vascular neointima formation. Nevertheless, the specific mechanisms of curcumin on VSMCs remain unclear. We confirmed that curcumin inhibits neointimal hyperplasia and demonstrates its ability to up-regulate miR-22 and downregulate SP1 in the injured vascular wall and VSMCs. In addition, the inhibition of miR-22 attenuated curcumin-induced inhibition on VSMC function. Thus, we revealed that curcumin inhibited the proliferation and migration of VSMCs and vascular neointimal hyperplasia partially through miR-22/SP1 signalling. We also demonstrated the involvement of miR-22 in VSMC function in response to vascular injury in vivo. The exact molecular mechanism that underlies the regulation of miR-22 expression remains unclear.

Nevertheless, a potential limitation should be noted. Currently, there is no uniform reference for curcumin concentration or dose for in vitro and in vivo experiments. In a study by Liu et al. (2014), the rats were pre-treated with low dose (50 mg/kg, intraperitoneal injection) and high dose (100 mg/kg, intraperitoneal injection) of curcumin for 5 days. In the study by Tamaddonfard et al. (2012), intraperitoneal injections of curcumin at 100 and 200 mg/kg were used. Gaedeke et al. (2005) used doses of 10 to 200 mg/kg body weight by intraperitoneal injection from days 3–5 after induction of disease, and the maximal inhibition doses were 50–100 mg/kg. Ray Hamidie et al. (2015) treated their rats with curcumin at 50 or 100 mg/kg/BW/day. Yu et al. (2011) used a dose of 50 mg/kg/day. He et al. (2010) used 50 mg/kg/day in 2-day-old neonatal Sprague-Dawley rats subjected to intracerebral injection of LPS. Samini et al. (2013) used an i.p. bolus of curcumin 50 or 100 mg/kg/day. Therefore, the present study selected a dose of 50 mg/kg/day.

Conclusion

We demonstrated that miR-22/SP1 is a novel regulatory pathway during the proliferation and migration of VSMCs in vitro. Moreover, curcumin was found to increase the level of miR-22 and inhibit VSMC function and neointima formation in vivo. This study demonstrated the potential of curcumin in clinical interventions for vascular diseases, particularly during restenosis after coronary intervention.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from the corresponding author, YTL, upon reasonable request.

References

Alexander MR, Owens GK. 2012. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annu Rev Physiol. 74:13–40.

Andan P, Kunnunakkara AB, Newman RA, Aggarwal BB. 2007. Bioavailability of curcumin: problems and promises. Mol Pharm. 4(6):807–818.

Cheng Y, Liu X, Yang J, Lin Y, Xu DZ, Lu Q, Deitch EA, Huo Y, Delphin ES, Zhang C. 2009. MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. Circ Res. 105(2):158–166.

Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, Lee TH, Miano JM, Ivey KN, Srivastava D. 2009. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature. 460(7256):705–710.

Curcio A, Torella D, Indolfi C. 2011. Mechanisms of smooth muscle cell proliferation and endothelial regeneration after vascular injury and stenting: approach to therapy. Circ J. 75(6):1287–1296.

Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A. 2009. Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. J Biol Chem. 284(6):3728–3738.

Deaton RA, Gan Q, Owens GK. 2009. Sp1-dependent activation of KLF4 is required for PDGF-BB-induced phenotypic modulation of smooth muscle. Am J Physiol Heart Circ Physiol. 296(4):H1027–1037.

Friese RS, Althsuler AE, Zhang K, Miramontes-Gonzalez JP, Hightower CM, Jirout ML, Salem RM, Gayen JR, Mahapatra NR, Biswas N, et al. 2013. MicroRNA-22 and promoter motif morphorphisms at the Chga locus in genetic hypertension: functional and therapeutic implications for gene expression and the pathogenesis of hypertension. Hum Mol Genet. 22(18):3624–3640.

Gaedeke J, Noble NA, Border WA. 2005. Curcumin blocks fibrosis in anti-Thy 1 glomerulonephritis through up-regulation of heme oxygenase 1. Kidney Int. 68(5):2042–2049.

Gurha P, Abreu-Goodger C, Wang T, Ramirez MO, Drumond AL, van Dongen S, Chen Y, Bartonieck N, Enright AJ, Lee B, et al. 2012. Targeted deletion of microRNA-22 promotes stress-induced cardiac dilatation and contractile dysfunction. Circulation. 125(22):2751–2761.

He LF, Chen HJ, Qian LH, Chen GY, Buzby JS. 2010. Curcumin protects pre-oligodendrocytes from activated microglia in vitro and in vivo. Brain Res. 1339:60–69.

Hewlings SJ, Kalman DS. 2017. Curcumin: a review of its effects on human health. Foods. 6(10):92.

Hu Y, Matkovich SJ, Hecker PA, Zhang Y, Edwards JR, Dorn GW. 2nd. 2012. Epitranscriptional orchestration of genetic reprogramming is an emergent property of stress-regulated cardiac microRNAs. Proc Natl Acad Sci USA. 109(48):19864–19869.

Huang SC, Wang M, Wu WB, Wang R, Cui J, Li W, Li ZL, Li W, Wang SM. 2017. Mir-22-3p inhibits arterial smooth muscle cell proliferation and migration and neointimal hyperplasia by targeting HMGB1 in arteriosclerosis obliterans. Cell Physiol Biochem. 42(6):2492–2506.

Huang ZP, Chen J, Seok HY, Zhang Z, Kataoka M, Hu X, Wang DZ. 2013. MicroRNA-22 regulates cardiac hypertrophy and remodeling in response to stress. Circ Res. 112(9):1234–1243.

Iaconetti C, De Rosa S, Polimeni A, Sorrentino S, Gareri C, Carino A, Sabatino J, Colangelo M, Curcio A, Indolfi C. 2015. Down-regulation of miR-23b induces phenotypic switching of vascular smooth muscle cells in vitro and in vivo. Cardiovasc Res. 107(4):522–533.

Jeong CW, Yoo KY, Lee SH, Jeong HJ, Lee CS, Kim SJ. 2012. Curcumin protects against regional myocardial ischemia/reperfusion injury through activation of RISK/GSK-3beta and inhibition of p38 MAPK and JNK. J Cardiovasc Pharmacol Ther. 17(4):387–394.

Kong LM, Liao CG, Zhang Y, Xu J, Li Y, Huang W, Zhang Y, Bian H, Chen ZN. 2014. A regulatory loop involving miR-22, Sp1, and c-Myc modulates CD147 expression in breast cancer invasion and metastasis. Cancer Res. 74(14):3764–3778.

Li B, Shi C, Li B, Zhao JM, Wang L. 2018. The effects of curcumin on HCT-116 cells proliferation and apoptosis via the miR-491/PEG10 pathway. J Cell Biochem. 119(4):3739–3751.

Liu K, Shen L, Wang J, Dong Y, Xu J, Li Y, Huang W, Zhang Y, Bian H, Chen ZN. 2014. A regulatory loop involving miR-22, Sp1, and c-Myc modulates CD147 expression in breast cancer invasion and metastasis. Cancer Res. 74(14):3764–3778.

Li B, Shi C, Li B, Zhao JM, Wang L. 2018. The effects of curcumin on HCT-116 cells proliferation and apoptosis via the miR-491/PEG10 pathway. J Cell Biochem. 119(4):3091–3098.

Li K, Shen L, Wang J, Dong Y, Xu J, Wu H, Shao H, Jing H. 2012. The preventative role of curcumin on the lung inflammatory response induced by cardiopulmonary bypass in rats. J Surg Res. 174(1):73–82.

Liu L, Zhang W, Wang L, Li Y, Tan B, Lu X, Deng Y, Zhang Y, Guo X, Mu J, et al. 2014. Curcumin prevents cerebral ischemia reperfusion injury via increase of mitochondrial biogenesis. Neurochem Res. 39(7):1332–1331.

Maheshwari RK, Singh AK, Gaddipati J, Srimal RC. 2006. Multiple biological activities of curcumin: a short review. Life Sci. 78(18):2087–2097.

Mintz GS, Popma JJ, Pichard AD, Kent KM, Satler LF, Wong C, Hong MK, Kovach JA, Leon MB. 1996. Arterial remodeling after coronary angioplasty: a serial intravascular ultrasound study. Circulation. 94(1):35–43.
Neely LA, Patel S, Garver J, Gallo M, Hackett M, McLaughlin S, Nadel M, Harris J, Gullans S, Rooke J. 2006. A single-molecule method for the quantitation of microRNA gene expression. Nat Methods. 3(1):41–46.

Pandey DP, Picard D. 2009. miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA. Mol Cell Biol. 29(13):3783–3790.

Paul P, Chakraborty A, Sarkar D, Langthasa M, Rahman M, Bari M, Singha RS, Malakar AK, Chakraborty S. 2018. Interplay between miRNAs and human diseases. J Cell Physiol. 233(3):2007–2018.

Ray Hamidie RD, Yamada T, Ishizawa R, Saito Y, Masuda K. 2015. Takeda E, Suzuki Y, Sato Y. 2016. Age-associated downregulation of vasohibin-1 in vascular endothelial cells. Aging Cell. 15(5):885–892.

Shishodia S. 2013. Molecular mechanisms of curcumin action: gene expression. Biofactors. 39(1):37–46.

Sun M, Estrov Z, Ji Y, Coombs KR, Harris DH, Kurzrock R. 2008. Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells. Mol Cancer Ther. 7(3):464–473.

Teiten MH, Dicato M, Diederich M. 2013. Curcumin as a regulator of epigenetic events. Mol Nutr Food Res. 57(9):1619–1629.

Torella D, Iaconetti C, Catalucci D, Ellison GM, Leone A, Waring CD, Bochicchio A, Vicianina C, Aquila I, Curcio A, et al. 2011. MicroRNA-133 controls vascular smooth muscle cell phenotypic switch in vitro and vascular remodeling in vivo. Circ Res. 109(8):880–893.

Wamhoff BR, Hoofnagle MH, Burns A, Sinha S, McDonald OG, Owens GK. 2004. A G/C element mediates repression of the SM22alpha promoter within phenotypically modulated smooth muscle cells in experimental atherosclerosis. Circ Res. 95(10):981–988.

Wang W, Sun W, Wang X. 2004. Intramuscular gene transfer of CGRP inhibits neointimal hyperplasia after balloon injury in the rat abdominal aorta. Am J Physiol Heart Circ Physiol. 287(4):H1582–H1589.

Wu T, Xiang Y, Ly V, Li D, Yu L, Guo R. 2017. miR-590-3p mediates the protective effect of curcumin on injured endothelial cells induced by angiotensin II. Am J Transl Res. 9(2):289–300.

Xiong J, Du Q, Liang Z. 2010. Tumor-suppressive microRNA-22 inhibits the transcription of E-box-containing c-Myc target genes by silencing c-Myc binding protein. Oncogene. 29(35):4980–4988.

Yang F, Chen Q, He S, Yang M, Maguire EM, An W, Afzal TA, Luong LA, Zhang L, Xiao Q. 2018. miR-22 is a novel mediator of vascular smooth muscle cell phenotypic modulation and neo-intima formation. Circulation. 137(17):1824–1841.

Yang X, Thomas DP, Zhang X, Culver BW, Alexander BM, Murdoch WJ, Rao MN, Tulis DA, Ren J, Sreejayan N. 2006. Curcumin inhibits platelet-derived growth factor-stimulated vascular smooth muscle cell function and injury-induced neo-intima formation. Arterioscler Thromb Vasc Biol. 26(1):85–90.

Yang Y, Duan W, Liang Z, Yi W, Yan J, Wang N, Li J, Chen W, Yu S, Jin Z, et al. 2013. Curcumin attenuates endothelial cell oxidative stress injury through Notch signaling inhibition. Cell Signal. 25(3):615–629.

Yao Y, Wang W, Li M, Ren H, Chen C, Wang J, Wang WE, Yang J, Zeng C. 2016. Curcumin exerts its anti-hypertensive effect by down-regulating the AT1 receptor in vascular smooth muscle cells. Sci Rep. 6:25579.

Yu WG, Xu G, Ren GJ, Xu X, Yuan HQ, Qi XL, Tian KL. 2011. Preventive action of curcumin in experimental acute pancreatitis in mice. Indian J Med Res. 134(5):717–724.

Yu YM, Lin HC. 2010. Curcumin prevents human aortic smooth muscle cells migration by inhibiting of MMP-9 expression. Nutr Metab Cardiovasc Dis. 20(2):125–132.

Zheng Y, Xu Z. 2014. MicroRNA-22 induces endothelial progenitor cell senescence by targeting AKT3. Cell Physiol Biochem. 34(5):1547–1555.