

Downregulated lncRNA UCA1 accelerates proliferation and migration of vascular smooth muscle cells by epigenetic regulation of MMP9

ZHIGANG XU¹, ZHENGQIN ZUO², DINGJUN DONG³, JUAN LIU³,
YONGQIAN TANG³, YUANYUN GU¹ and HAI LIU³

¹Department of Geriatrics and ²Ultrasonography Laboratory, Chinese Medicine Hospital Affiliated to Southwest Medical University, Luzhou, Sichuan 646000; ³Department of Cardiology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiangyang, Hubei 441021, P.R. China

Received September 27, 2019; Accepted November 25, 2019

DOI: 10.3892/etm.2020.8639

Abstract. Function of long non-coding RNA urothelial carcinoma antigen 1 (lncRNA UCA1) in regulating the proliferative and migratory abilities of vascular smooth muscle cells (VSMCs) by mediating matrix metalloproteinase-9 (MMP9) level were elucidated. After treatment with different concentrations of ox-LDL for different time points, lncRNA UCA1 level in VSMCs was determined by quantitative real-time polymerase chain reaction (qRT-PCR). Subcellular distribution of UCA1 was analyzed. Proliferative and migratory abilities of VSMCs transfected with pcDNA-UCA1 were assessed. Protein level of MMP9 in HA-VSMCs treated with different concentrations of ox-LDL for different time points was also determined. The potential interaction between UCA1 and enhancer of zeste homolog 2 (EZH2) was identified by RNA immunoprecipitation (RIP) assay. Recruitment ability of EZH2 to MMP9 promoter region influenced by UCA1 was determined by Chromatin immunoprecipitation (ChIP) assay. Finally, the potential function of MMP9 in UCA1-mediated cellular behavior of VSMCs was explored. UCA1 was time-dependently and dose-dependently upregulated in VSMCs by ox-LDL treatment. Proliferative and migratory abilities of VSMCs were enhanced by treatment of 100 mg/l ox-LDL for 48 h, which were further reduced after transfection of pcDNA-UCA1. Subcellular distribution analysis showed that UCA1 was mainly distributed in the nucleus. Protein level of MMP9 was gradually elevated with the treatment of increased concentrations of ox-LDL in VSMCs. Its level was down-regulated by transfection of pcDNA-UCA1 in VSMCs. The

interaction between UCA1 and EZH2 was confirmed by RIP assay. Transfection of pcDNA-UCA1 stimulated the binding of EZH2 on MMP9 promoter region. Finally, overexpression of MMP9 reversed the decreased proliferative and migratory abilities in ox-LDL-treated VSMCs overexpressing UCA1. Downregulated UCA1 accelerates VSMCs to proliferate and migrate through negatively regulating MMP9 level.

Introduction

Vascular smooth muscle cells (VSMCs) are vital cells that maintain normal physiological functions of blood vessels. Under normal conditions, VSMCs are non-proliferative contractile type. However, they are stimulated to proliferate in the presence of vascular injury and some bioactive substances (i.e. nitric oxide products, angiotensin II and platelet growth factor). Proliferative VSMCs synthesize and secrete vasoactive substances and growth factors, thus leading to thickening of blood vessels, luminal stenosis and vascular remodeling (1). Phenotype conversion and proliferation stimulation of VSMCs are the key factors in the development of vascular proliferative diseases, such as hypertension and atherosclerosis (2,3).

Long non-coding RNA (lncRNA) is a class of ncRNAs synthesized by RNA polymerase II over 200 nucleotides long. In generally, lncRNAs are classified into five subtypes, namely antisense lncRNAs, intronic transcripts, large intergenic noncoding RNAs, promoter-associated lncRNAs and UTR-associated lncRNAs (4,5). It is reported that certain lncRNAs are able to influence the phenotypes of VSMCs and further affect the occurrence of atherosclerosis (6,7). lncRNA UCA1 (urothelial carcinoma antigen 1) was initially discovered by Wang *et al* (8). UCA1 locates on 19p13.12, and is commonly expressed in embryonic tissues. Han *et al* (9) found that UCA1 is highly expressed in colorectal cancer tissues, which is closely related to tumor size, depth of invasion and poor tissue differentiation. A recent study demonstrated the ability of UCA1 in mediating the proliferative and migratory capacities of VSMCs (10).

Matrix metalloproteinases (MMPs), known as matrix metalloproteinases, are calcium-dependent zinc-containing

Correspondence to: Dr Hai Liu, Department of Cardiology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, 136 Jingzhou Street, Xiangyang, Hubei 441021, P.R. China
E-mail: jideng19600314@163.com

Key words: VSMCs, UCA1, MMP9, proliferation, migration

endopeptidases. They are capable of degrading components of the extracellular matrix (ECM), including laminin, collagen, and fibronectin (11). Currently, at least 26 members of the MMPs family have been discovered. Among them, MMP9 is closely related to cerebrovascular system (12). MMP9, also known as gelatinase B or 92 kDa gelatinase, locates on 16q 11.2-13.1 and contains 13 exons. The basic structure of MMP9 consists of a signal peptide region, amino-terminal propeptide, the zinc-binding catalytic domain, the carboxyl-terminal hemopexin-like domain and the hinge region (13). A relevant study has demonstrated that MMP9 downregulation suppressed chlamydia pneumonia infection-induced migration of VSMCs (14). This study mainly investigated the potential function of UCA1 in ox-LDL-treated cellular phenotype changes of VSMCs through regulating MMP9, thus providing novel directions in the treatment of vascular diseases.

Materials and methods

Cell culture and induction. VSMCs were provided by Cell Bank (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) (HyClone) containing 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), 100 µg/ml penicillin and 0.1 µg/ml streptomycin, at 37°C, in a 5% CO₂ incubator. Fourth to fifth generation VSMCs were selected for treatment with ox-LDL.

Cell transfection. Cells were inoculated in 6-well plates with 2x10⁵ cells per well. At 80% confluence, cells were transfected using Lipofactamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Medium containing 2 µg/ml puromycin was replaced 48 h later, and continued for 72 h of culture. Positive colonies were selected and amplified for *in vitro* experiments.

Quantitative real-time polymerase chain reaction (qRT-PCR). Extraction of total RNA in cells was performed using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and subjected to reverse transcription. The extracted complementary deoxyribose nucleic acid (cDNA) was applied for PCR using SYBR Green method. Primer sequences were as follows: UCA1, forward: 5'-CTCTCCATTGGGTTCACCATTC-3' and reverse: 5'-GCGGCAGGTCTTAAGAGATGAG-3'; MMP9, forward: 5'-CGATGCCTGCAACGTGAAC-3' and reverse: 5'-AGAGCCGCTCCTCAAAGACC-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-TGAAGGTCGGAGTCAACGG-3' and reverse: 5'-CCTGGAAGATGGTGATGCG-3'.

Cell Counting Kit-8 (CCK-8). Cells were seeded in a 96-well plate and cultured overnight. Absorbance (A) at 490 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories) for depicting the viability curves.

Transwell migration assay. Cells transfected for 48 h were adjusted to the dose of 1.0x10⁵ cells/ml and subjected to serum starvation for 12 h. Then, 200 µl/well suspension was applied to the upper Transwell chamber (Merck KGaA). In the lower chamber, 700 µl of medium containing 10% FBS was applied. After 48 h of incubation, cells migrated to the lower chamber

were subjected to fixation in methanol for 15 min, crystal violet staining for 20 min and cell counting using a microscope. Penetrating cells were counted in 5 randomly selected fields per sample.

Western blotting. Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Pierce; Thermo Fisher Scientific, Inc.). Protein sample was loaded for electrophoresis and transferred on polyvinylidene fluoride (PVDF) membranes (Merck KGaA). Membranes were blocked in 5% skim milk for 2 h, and subjected to incubation with primary and secondary antibodies. Bands were exposed by electrochemiluminescence (ECL) and analyzed by Image Software (National Institutes of Health).

Determination of subcellular distribution. Cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen; Thermo Fisher Scientific, Inc.) and subjected to qRT-PCR. 18s was the internal reference of nucleus and U1 was that of the cytoplasm.

RNA immunoprecipitation (RIP). Cells were treated according to the procedures of Millipore Magna RIPTM RNA-Binding Protein Immunoprecipitation kit. Cell lysate was incubated with anti-EZH2 (enhancer of zeste homolog 2), or anti-IgG antibody at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/ml proteinase K containing 0.1% SDS to extract RNA. The magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to mRNA level determination using qRT-PCR.

Chromatin immunoprecipitation (ChIP). Cells were subjected to 10 min cross-link with 1% formaldehyde at room temperature into small fractions with 200-1000 bp. Subsequently, cells were lysed and sonicated for 30 min. Finally, the sonicated lysate was immuno-precipitated with anti-EZH2, anti-H3K27me3 or anti-IgG. Purified immunoprecipitated chromatin were subjected to qRT-PCR.

Statistical analysis. Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp.) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of UCA1 in VSMCs undergoing ox-LDL treatment. QRT-PCR data showed that UCA1 level was gradually reduced after 50 and 100 mg/l ox-LDL treatment in VSMCs for 48 h (Fig. 1A). With the prolongation of 100 mg/l ox-LDL treatment, UCA1 was downregulated at 24 and 48 h (Fig. 1B). It is indicated that UCA1 was dose-dependently and time-dependently downregulated by ox-LDL treatment. Transfection of pcDNA-UCA1 sufficiently upregulated UCA1 level in VSMCs, showing great transfection efficacy (Fig. 1C). CCK-8 assay showed increased viability in VSMCs undergoing 100 mg/l ox-LDL treatment for 48 h, which was reversed

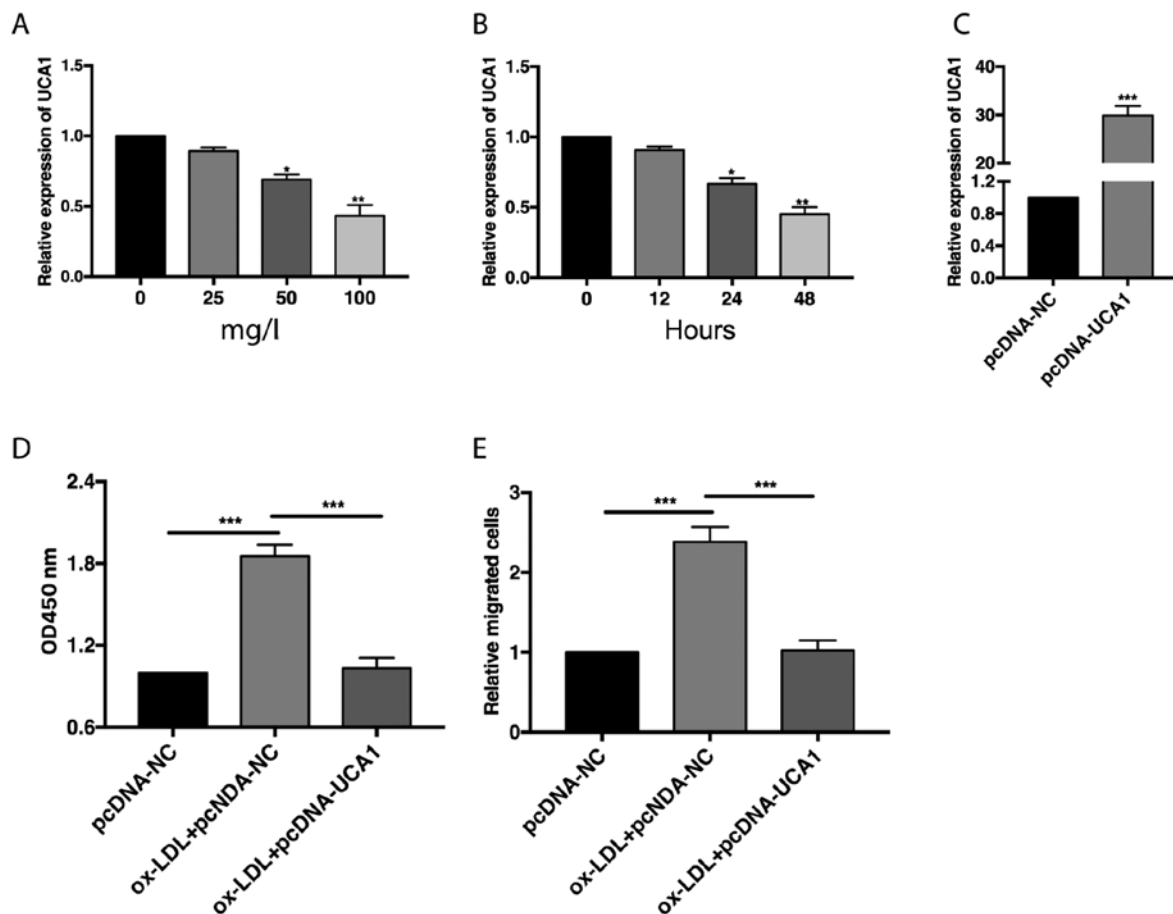


Figure 1. Downregulation of UCA1 in VSMCs undergoing ox-LDL treatment. (A) Relative level of UCA1 in VSMCs induced with 0, 25, 50 and 100 mg/l ox-LDL for 48 h. (B) Relative level of UCA1 in VSMCs induced with 100 mg/l ox-LDL for 0, 12, 24 and 48 h. (C) Transfection efficacy of pcDNA-UCA1 in VSMCs. (D) CCK-8 assay showed viability in VSMCs transfected with pcDNA-NC, ox-LDL + pcDNA-NC or ox-LDL + pcDNA-UCA1. (E) Relative number of migratory VSMCs transfected with pcDNA-NC, ox-LDL + pcDNA-NC or ox-LDL + pcDNA-UCA1. UCA1, urothelial carcinoma antigen 1; VSMCs, vascular smooth muscle cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

by transfection of pcDNA-UCA1 (Fig. 1D). Similarly, relative number of migratory VSMCs increased by 100 mg/l ox-LDL treatment for 48 h, and was further reduced after overexpression of UCA1 (Fig. 1E). It is suggested that UCA1 suppressed the proliferative and migratory abilities of VSMCs.

UCA1 negatively regulates MMP9 level. Subcellular distribution analysis indicated that UCA1 was mainly enriched in the nucleus (Fig. 2A). Treatment of ox-LDL in VSMCs gradually upregulated protein level of MMP9 in a concentration-dependent manner (Fig. 2B). In addition, transfection of pcDNA-UCA1 markedly downregulated MMP9 level (Fig. 2C). RIP assay pointed out higher enrichment of UCA1 in anti-EZH2 relative to anti-IgG (Fig. 2D). Transfection of si-EZH2 markedly upregulated MMP9 level in VSMCs (Fig. 2E). Furthermore, higher immunoprecipitants of EZH2 and H3K27me3 were shown in VSMCs overexpressing UCA1 (Fig. 2F). It is suggested that UCA1 recruited EZH2 to negatively mediate the PTEN level.

MMP9 partially reverses the biological role of UCA1. Transfection of pcDNA-MMP9 remarkably upregulated mRNA and protein level of MMP in VSMCs (Fig. 3A and B). Overexpression of UCA1 in ox-LDL-treated VSMCs attenuated

their proliferative and migratory abilities, but were further reversed by MMP overexpression (Fig. 3C and D). Hence, it is believed that UCA1 suppressed proliferative and migratory abilities of VSMCs by negatively regulating the MMP9 level.

Discussion

Dysfunction of VSMCs contributes to the occurrence and development of cardiovascular diseases (15,16). In recent years, the morbidity and mortality of cardiovascular diseases, including hypertension, atherosclerosis and ischemic encephalopathy have been enhanced each year. VSMCs and vascular endothelial cells are important components of blood vessels. The former are located in the tunica media vasorum and the latter are distributed in the tunica intima vasorum. Under normal circumstances, VSMCs are differentiated and mature (contractile type), which maintains the normal contractile function of the arterial wall and regulates blood pressure. After vascular endothelium damage or surrounding microenvironment changes, multiple activated pathways stimulate the contractile type of VSMCs into synthetic type. At this time, VSMCs are prone to proliferate and migrate, which accelerate the deposition of ECMs in blood vessels and lead to vascular remodeling (17,18).

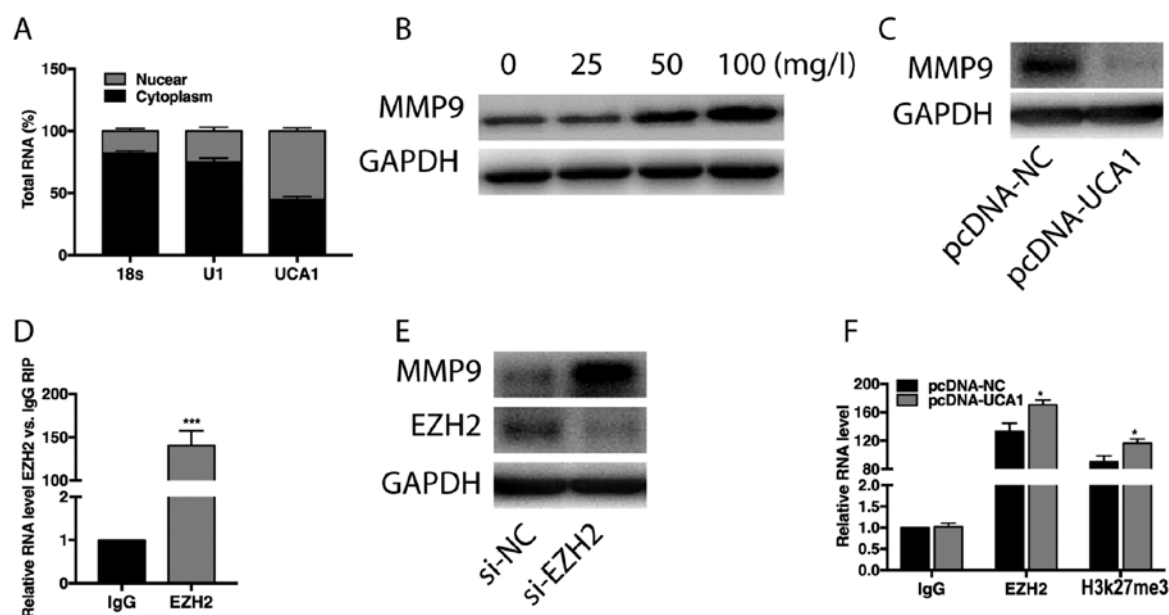


Figure 2. UCA1 negatively regulates MMP9 level. (A) Subcellular distribution of UCA1 in nuclear and cytoplasmic fractions of VSMCs. 18s and U1 are internal reference for cytoplasm and nucleus, respectively. (B) Relative level of MMP9 in VSMCs induces with 0, 25, 50 and 100 mg/l ox-LDL for 48 h. (C) Relative level of MMP9 in VSMCs transfected with pcDNA-NC or pcDNA-UCA1. (D) RIP assay showed the enrichment of UCA1 in anti-IgG or anti-EZH2. (E) Protein levels of MMP9 and EZH2 in VSMCs transfected with si-NC or si-EZH2. (F) ChIP assay shows the immunoprecipitants of IgG, EZH2 and H3K27me3 in VSMCs transfected with pcDNA-NC or pcDNA-UCA1. UCA1, urothelial carcinoma antigen 1; VSMCs, vascular smooth muscle cells; MMP9, matrix metalloproteinase-9; EZH2, enhancer of zeste homolog 2; RIP, RNA immunoprecipitation; ChIP, Chromatin immunoprecipitation. * $P < 0.05$, *** $P < 0.001$.

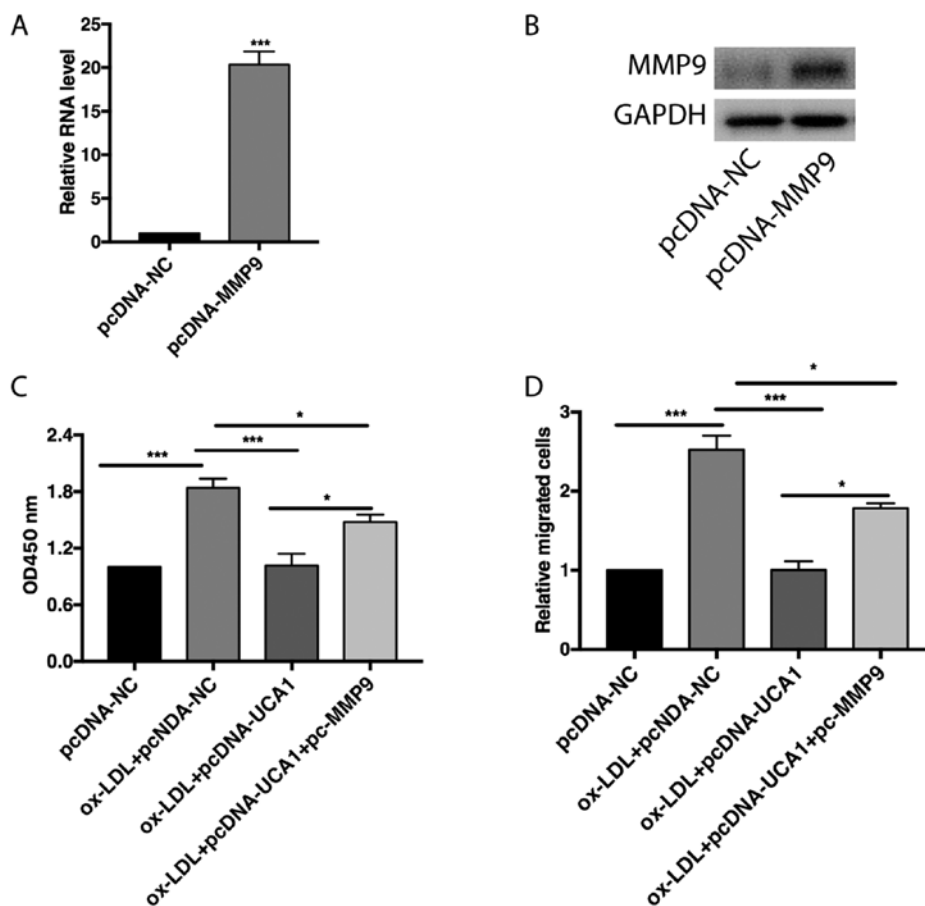


Figure 3. MMP9 partially reverses the biological role of UCA1. (A) Relative level of MMP9 in VSMCs transfected with pcDNA-NC or pcDNA-MMP9. (B) Protein level of MMP9 in VSMCs transfected with pcDNA-NC or pcDNA-MMP9. (C) CCK-8 assay shows the viability in VSMCs transfected with pcDNA-NC, ox-LDL + pcDNA-NC, ox-LDL + pcDNA-UCA1 or ox-LDL + pcDNA-UCA1 + pcDNA-MMP9. (D) Relative number of migratory VSMCs transfected with pcDNA-NC, ox-LDL + pcDNA-NC, ox-LDL + pcDNA-UCA1 or ox-LDL + pcDNA-UCA1 + pcDNA-MMP9. UCA1, urothelial carcinoma antigen 1; VSMCs, vascular smooth muscle cells; MMP9, matrix metalloproteinase-9; CCK-8, Cell Counting Kit-8. * $P < 0.05$, *** $P < 0.001$.

lncRNAs are defined as transcripts without protein-encoding ability. They are able to influence tumorigenesis through acting on multiple pathways. Abnormally expressed lncRNAs can be detected in the serum, urine or tumor cells in tumor patients. They present specific expression patterns in different stages of tumor diseases and different types of tissues. Therefore, lncRNAs could be utilized as diagnostic hallmarks for tumors (19). It is indicated that downregulation of lncRNA RNCR3 accelerates the occurrence of atherosclerosis, elevates blood lipid levels and stimulates inflammatory response. Moreover, the differentiation and migration of endothelial cells and VSMCs are suppressed, while their apoptotic abilities are enhanced (20). In this study, UCA1 was gradually downregulated with the prolongation of increased concentrations of ox-LDL treatment. Overexpression of UCA1 attenuated the proliferative and migratory abilities of VSMCs.

MMPs and their tissue inhibitors are a class of zinc-containing enzymes that degrade ECMs and remodel ECM proteins. MMPs are mainly produced and released by smooth muscle cells, fibroblasts, and inflammatory cells. MMP9 belongs to gelatinase, which degrades both elastin and collagen (21). Relevant studies have shown that MMP9 influences familial aortic dissection by activating TGF- β /Smad pathway (22). Specifically, MMP9 is able to regulate the balance of ECM synthesis and degradation, systolic function of VSMCs and normal function and structure of the aortic wall. lncRNA MEG8 is reported to affect the proliferative ability of VSMCs through targeting PPAR α (23). Consistently, this study demonstrated that UCA1 suppressed the proliferative and migratory abilities of VSMCs through regulating MMP9. Our conclusions may lay a solid foundation for VSMC research and the application in clinical practice.

In conclusion, downregulated UCA1 accelerates VSMCs to proliferate and migrate through negatively regulating the MMP9 level.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZX and HL designed the study and performed the experiments, DD, ZZ and JL collected the data, YT and YG analyzed the data, ZX and HL prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Kil JS, Jeong SO, Chung HT and Pae HO: Piceatannol attenuates homocysteine-induced endoplasmic reticulum stress and endothelial cell damage via heme oxygenase-1 expression. *Amino Acids* 49: 735-745, 2017.
- Ren XS, Tong Y, Ling L, Chen D, Sun HJ, Zhou H, Qi XH, Chen Q, Li YH, Kang YM, *et al*: NLRP3 gene deletion attenuates angiotensin II-induced phenotypic transformation of vascular smooth muscle cells and vascular remodeling. *Cell Physiol Biochem* 44: 2269-2280, 2017.
- Wei HJ, Xu JH, Li MH, Tang JP, Zou W, Zhang P, Wang L, Wang CY and Tang XQ: Hydrogen sulfide inhibits homocysteine-induced endoplasmic reticulum stress and neuronal apoptosis in rat hippocampus via upregulation of the BDNF-TrkB pathway. *Acta Pharmacol Sin* 35: 707-715, 2014.
- Kung JT, Colognori D and Lee JT: Long noncoding RNAs: Past, present, and future. *Genetics* 193: 651-669, 2013.
- Li FP, Lin DQ and Gao LY: lncRNA TUG1 promotes proliferation of vascular smooth muscle cell and atherosclerosis through regulating miRNA-21/PTEN axis. *Eur Rev Med Pharmacol Sci* 22: 7439-7447, 2018.
- Ballantyne MD, Pinel K, Dakin R, Vesey AT, Diver L, Mackenzie R, Garcia R, Welsh P, Sattar N, Hamilton G, *et al*: Smooth muscle enriched long noncoding RNA (SMILR) regulates cell proliferation. *Circulation* 133: 2050-2065, 2016.
- Yao QP, Xie ZW, Wang KX, Zhang P, Han Y, Qi YX and Jiang ZL: Profiles of long noncoding RNAs in hypertensive rats: Long noncoding RNA XR007793 regulates cyclic strain-induced proliferation and migration of vascular smooth muscle cells. *J Hypertens* 35: 1195-1203, 2017.
- Wang XS, Zhang Z, Wang HC, Cai JL, Xu QW, Li MQ, Chen YC, Qian XP, Lu TJ, Yu LZ, *et al*: Rapid identification of UCA1 as a very sensitive and specific unique marker for human bladder carcinoma. *Clin Cancer Res* 12: 4851-4858, 2006.
- Han Y, Yang YN, Yuan HH, Zhang TT, Sui H, Wei XL, Liu L, Huang P, Zhang WJ and Bai YX: UCA1, a long non-coding RNA up-regulated in colorectal cancer influences cell proliferation, apoptosis and cell cycle distribution. *Pathology* 46: 396-401, 2014.
- Tian S, Yuan Y, Li Z, Gao M, Lu Y and Gao H: lncRNA UCA1 sponges miR-26a to regulate the migration and proliferation of vascular smooth muscle cells. *Gene* 673: 159-166, 2018.
- Turner RJ and Sharp FR: Implications of MMP9 for blood brain barrier disruption and hemorrhagic transformation following ischemic stroke. *Front Cell Neurosci* 10: 56, 2016.
- Yang R, Zhang Y, Huang D, Luo X, Zhang L, Zhu X, Zhang X, Liu Z, Han JY and Xiong JW: Miconazole protects blood vessels from MMP9-dependent rupture and hemorrhage. *Dis Model Mech* 10: 337-348, 2017.
- Lenglet S, Montecucco F, Mach F, Schaller K, Gasche Y and Copin JC: Analysis of the expression of nine secreted matrix metalloproteinases and their endogenous inhibitors in the brain of mice subjected to ischaemic stroke. *Thromb Haemost* 112: 363-378, 2014.
- Ma L, Zhang L, Wang B, Wei J, Liu J and Zhang L: Berberine inhibits Chlamydia pneumoniae infection-induced vascular smooth muscle cell migration through downregulating MMP3 and MMP9 via PI3K. *Eur J Pharmacol* 755: 102-109, 2015.
- McDonald RA, Hata A, MacLean MR, Morrell NW and Baker AH: MicroRNA and vascular remodelling in acute vascular injury and pulmonary vascular remodelling. *Cardiovasc Res* 93: 594-604, 2012.
- Leeper NJ and Maegdefessel L: Non-coding RNAs: Key regulators of smooth muscle cell fate in vascular disease. *Cardiovasc Res* 114: 611-621, 2018.

17. Ha JM, Yun SJ, Kim YW, Jin SY, Lee HS, Song SH, Shin HK and Bae SS: Platelet-derived growth factor regulates vascular smooth muscle phenotype via mammalian target of rapamycin complex 1. *Biochem Biophys Res Commun* 464: 57-62, 2015.
18. Yang F, Chen Q, He S, Yang M, Maguire EM, An W, Afzal TA, Luong LA, Zhang L and Xiao Q: miR-22 is a novel mediator of vascular smooth muscle cell phenotypic modulation and neointima formation. *Circulation* 137: 1824-1841, 2018.
19. Su YJ, Yu J, Huang YQ and Yang J: Circulating long noncoding RNA as a potential target for prostate cancer. *Int J Mol Sci* 16: 13322-13338, 2015.
20. Shan K, Jiang Q, Wang XQ, Wang YN, Yang H, Yao MD, Liu C, Li XM, Yao J, Liu B, *et al*: Role of long non-coding RNA-RNCR3 in atherosclerosis-related vascular dysfunction. *Cell Death Dis* 7: e2248, 2016.
21. Wang L, Zhang J, Fu W, Guo D, Jiang J and Wang Y: Association of smooth muscle cell phenotypes with extracellular matrix disorders in thoracic aortic dissection. *J Vasc Surg* 56: 1698-1709, 2012.
22. Dekkers BG, Naeimi S, Bos IS, Menzen MH, Halayko AJ, Hashjin GS and Meurs H: L-thyroxine promotes a proliferative airway smooth muscle phenotype in the presence of TGF-beta1. *Am J Physiol Lung Cell Mol Physiol* 308: L301-L306, 2015.
23. Zhang B, Dong Y and Zhao Z: LncRNA MEG8 regulates vascular smooth muscle cell proliferation, migration and apoptosis by targeting PPAR α . *Biochem Biophys Res Commun* 510: 171-176, 2019.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.